GUT ENDODERM IS INVOLVED IN THE TRANSFER OF LEFT-RIGHT ASYMMETRY FROM THE NODE TO THE LATERAL PLATE MESODERM IN THE MOUSE EMBRYO

by

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ABSTRACT

Left-right (LR) determination during embryogenesis is critical for the correct positioning of the various visceral organs, such as the lungs and the heart. In establishing LR asymmetry in the mouse, the initial LR signal that is determined in the ciliated embryonic node is transferred to the lateral plate mesoderm (LPM). The cellular and molecular mechanisms for this signal transfer have not been well characterized. Here, we studied the role of endoderm cells in this process by analyzing mouse *Sox17* null mutant embryos as a model that develops endoderm-specific defects. *Sox17* mutant embryos showed no expression or significantly reduced expression of LR asymmetric genes in the left LPM. A series of experiments revealed the importance of intercellular communication through gap junctions in endoderm cells for LR signal transfer from the node. We also found that SOX17 function is essential not only to form complete epithelial structures that express CX43 and localize connexin proteins on the cell membrane but also to turn off *Nodal* gene expression in differentiating endoderm cells through a *Nodal* endoderm-specific enhancer.

For Yukio

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CHAPTER 1

INTRODUCTION

Internal left-right asymmetry

The vertebrate body plan is externally symmetric, implying that the left and right sides of the body are mirror images of each other. However, there is left-right (LR) asymmetry in the organization of internal organs in the body. The internal organs can be classified into unpaired organs such as the heart, liver, spleen and the stomach, whereas the paired organs include the lungs, the bronchi and the atrial appendages. During development, the arrangement of unpaired internal organs occurs due to movement of these organs along the LR axis from the midline to their final positions. The paired organs on the other hand, form distinct morphological forms on the left and the right sides, such as the left and right lungs in humans comprising two and three lobes, respectively. The LR differences in the organization of the internal body plan follows a definite pattern on either side of the midline and is known as *situs solitus* (Figure 1.1A; Capdevila et al., 2000).

Clinical manifestation of left-right defects

Defects in the generation of normal LR asymmetry result in a range of laterality abnormalities, collectively called heterotaxia. Three different types of anatomical heterotaxia have been recognized and are caused by the failure of internal organs to maintain their relative positions with respect to each other. The first type of LR defect is

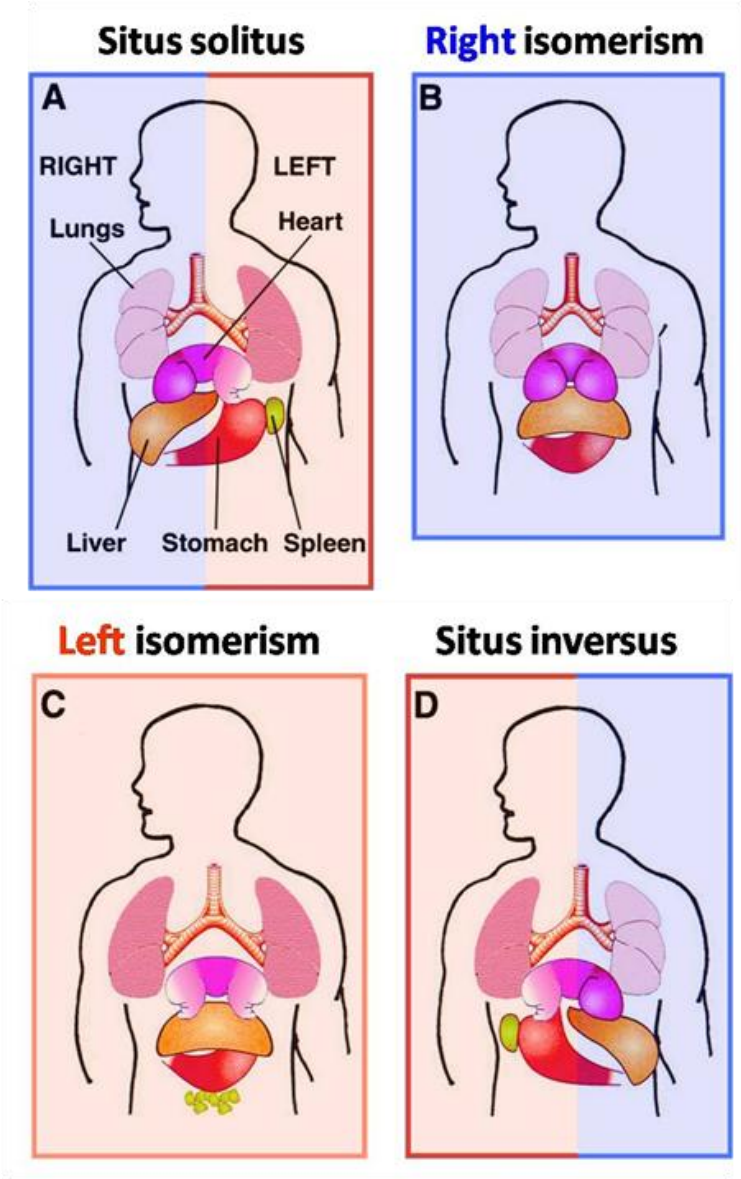


Figure 1.1 Asymmetric organization of internal organs. The normal pattern of left-right differences is called *situs solitus* (A). In *right isomerism* (B), both sides adopt the right side features of the normal pattern, for example, three lung lobes in the left side. In *left isomerism* (C), both sides adopt the left side features of the normal pattern, for example, more than one spleen and two lung lobes on the right side. *Situs inversus* results in complete mirror image inversion of the normal LR differences (D) (Capdevilla et al., 2000).

situs inversus totalis, which results in a complete mirror image inversion of all internal organs and occurs at the rate of about 1 in 6000-8000 births (Figure 1.1D; Peeters and Devriendt, 2006). The second type of defect is *situs ambiguus*, where at least one organ shows abnormal positioning with respect to the normal LR orientation. This condition involves defects in the placement of unpaired organs, such as the heart and occurs in 1/10000 births. The third type is referred to as *isomerism*, in which the LR asymmetry of one or more paired organs, such as bronchi and the lungs, occurs as mirror images of the normal condition. For example, in left isomerism, both lungs have two lobes, whereas in right isomerism, the lungs are trilobed (Figure 1.1B, C).

Several laterality disorders arise that are associated with organ malformations and functional impairments as a result of these LR defects during development. *Situs ambiguus* and *isomerism* are frequently associated with complex congenital heart disorders, spleen and gastrointestinal anomalies such as right-sided stomach, intestinal malrotation, midline-located liver and poly or asplenia (Kosaki and Casey, 1998; Kathiriya and Srivastava, 2000). The cardiac defects that arise due to *situs ambiguus* include transposition of the great arteries, double outlet right ventricle, double inlet left ventricle, atrioventricular septal defects and total anomalous pulmonary venous connection. These severe congenital defects incur huge costs to the healthcare system and illustrate the importance of establishing the correct orientation of internal organs with respect to each other. Thus, it is critical to investigate the mechanisms that regulate LR asymmetry to gain insights into how these abnormalities develop during embryogenesis.

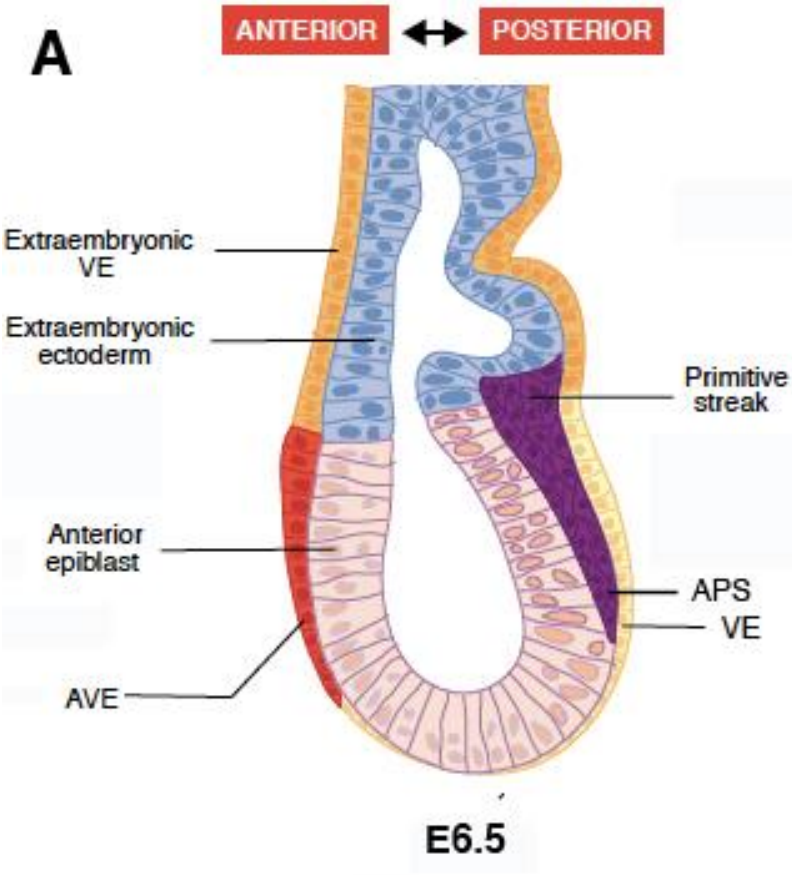
Early mouse embryogenesis and left-right determination

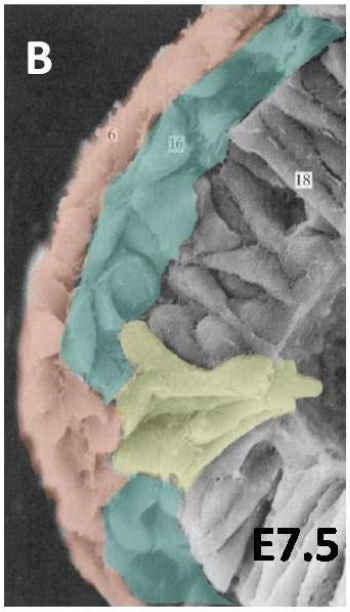
Patterning of vertebrate embryos involves establishment of three body axes: anterior-posterior (AP), dorso-ventral (DV) and the left-right (LR) axis. Cell proliferation of the mouse preimplantation stage results in the blastocyst formation at embryonic day 3.5 to 4.5 (E3.5-E4.5). The A-P axis is determined by the migration of a specialized group of cells called the anterior visceral endoderm (AVE) positioned at the distal end of the embryo at E5.5 in the proximal direction to reach the future anterior side of the embryo at E6.0 (Figure 1.2A).

Gastrulation begins at E6.5 at the opposite side of the AVE and is marked by the appearance of the primitive streak, identifying the posterior side of the embryo (Robb and Tam, 2004; Tam et al., 2007). At this stage, the cells of the future dorsal side of the embryo lie facing the blastocyst cavity and the future ventral cells lie on the outside facing the exterior. The primitive streak arises from the epithelial-mesenchymal transition (EMT) of the posterior epiblast cells and elongates from the proximal to the distal region during E6.5 to E7.0 (Figure 1.2B). Two types of cells emerge from the primitive streak: mesoderm cells that are sandwiched between the epiblast cells and the outer endoderm layer, and definitive endoderm cells that intercalate in between cells of the outer visceral endoderm layer (Figure 1.2B, C). Definitive (or embryonic) endoderm cells give rise to the endoderm lineage of the embryo proper, while the visceral endoderm cells form the supporting extraembryonic layer that later forms the yolk sac. Thus, the AP and the DV axes are already specified prior to the initiation of the LR axis.

The LR determination following establishment of the AP and DV axis can be divided into three phases (Figure 1.3; Hamada et al., 2002; Robb and Tam, 2004). First,

Figure 1.2 Early mouse embryogenesis and the origin of definitive endoderm. (A) Migration of the anterior visceral endoderm (AVE, colored red) to the proximal region determines the anterior side at E6.0, following which the primitive streak (colored purple) arises at the opposite end (posterior side) at E6.5 forming the A-P axis. APS, anterior primitive streak; VE, visceral endoderm. (B) Scanning Electron Microscope image of a cross-section of the posterior side (at E7.5) showing the epithelial to mesenchymal transition at the primitive streak (yellow) to form mesoderm (green) as the middle layer and definitive endoderm on the outside (red). (C) At E8.0, the definitive endoderm (red) arising from the primitive streak migrates from the posterior to the anterior side to cover the entire embryo (Adapted from Lu et al., 2001; Laboratory studies of vertebrate and invertebrate embryos by Schoenwolf, 2000; SEM atlas of Mouse development by, Tatsuo et al., 2003 Iwanami publishers, Japan).


A
ANTERIOR
POSTERIOR
Extraembryonic VE
Extraembryonic ectoderm
Primitive streak
Anterior epiblast
APS
VE
AVE
E6.5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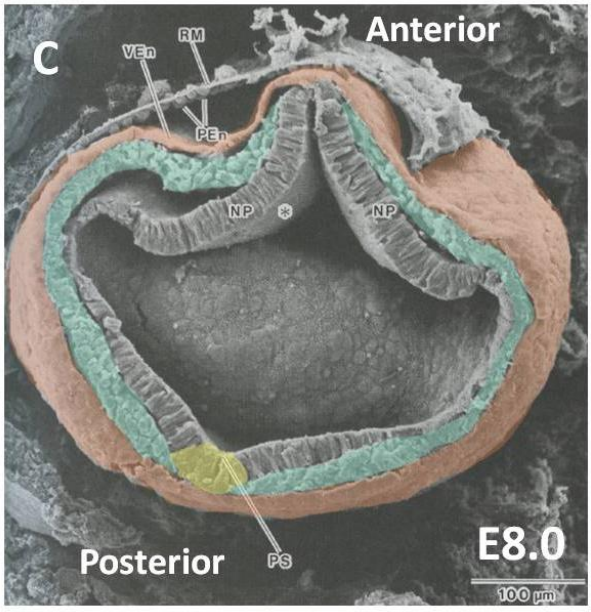
E7.5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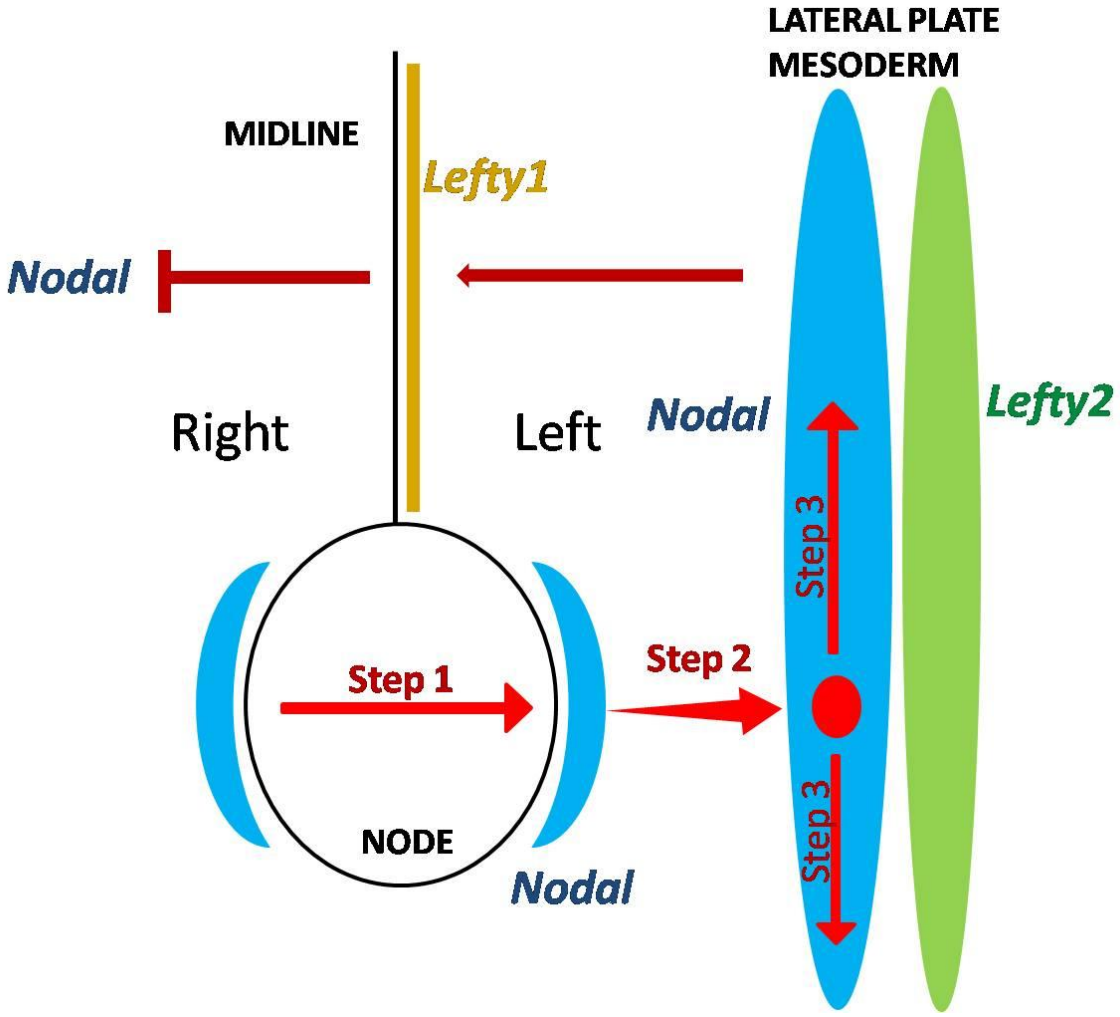


Figure 1.3. The three steps of LR determination. Step 1: Symmetry breaking in the node. Step 2: Transfer of LR signal from the node to the LPM. Step 3: Expansion of *Nodal* and *Lefty2* expression in the left LPM. *Nodal* expression in the left LPM induces *Lefty1* expression in the midline that prevents activation of *Nodal* expression on the right side.

the initial breakage of bilateral symmetry occurs in the node, a midline-located embryonic structure, located along the midline at 8.0 dpc (days post coitum). Next, the LR signal generated in the node is transferred to the lateral plate mesoderm (LPM), the derivatives of which surround the gut tube and anchor the internal organs in their respective positions. In the third step, asymmetric gene expression of signaling molecules in the LPM is initiated and expands to cover the entire LPM. These steps are discussed in further detail below:

Symmetry breaking in the node

The first event in LR determination occurs in the node, an organizer structure composed of a specialized group of cells that are lined with cilia on their ventral surface projecting into the extracellular space (Figure 1.4A, B; Sulik et al., 1994). The cilia are tilted posteriorly and rotate unidirectionally in a clockwise direction (Nonaka et al., 2005; Okada et al., 2005). This movement causes a leftward laminar flow of the extracellular fluid, as visualized by leftward movement of fluorescent beads over the node surface in culture conditions and is known as "nodal flow" (Figure 1.4C; Nonaka et al., 1998). Artificial reversal of nodal flow by forced movement of the extracellular fluid in the rightward direction causes inversion of LR symmetry, suggesting a central role for nodal flow in the symmetry breaking step (Nonaka et al., 2002). The significance of nodal flow for LR determination is well established; however, the mechanism by which nodal flow generates the LR signal on the left side is not completely understood (Okada et al., 1999). *Nodal* is expressed in the perinodal (or crown) cells and is required for the asymmetric gene expression in the LPM (Brennan et al., 2002; Saijoh et al., 2003). So far, NODAL is the only known signaling molecule in the node that has been shown genetically to be

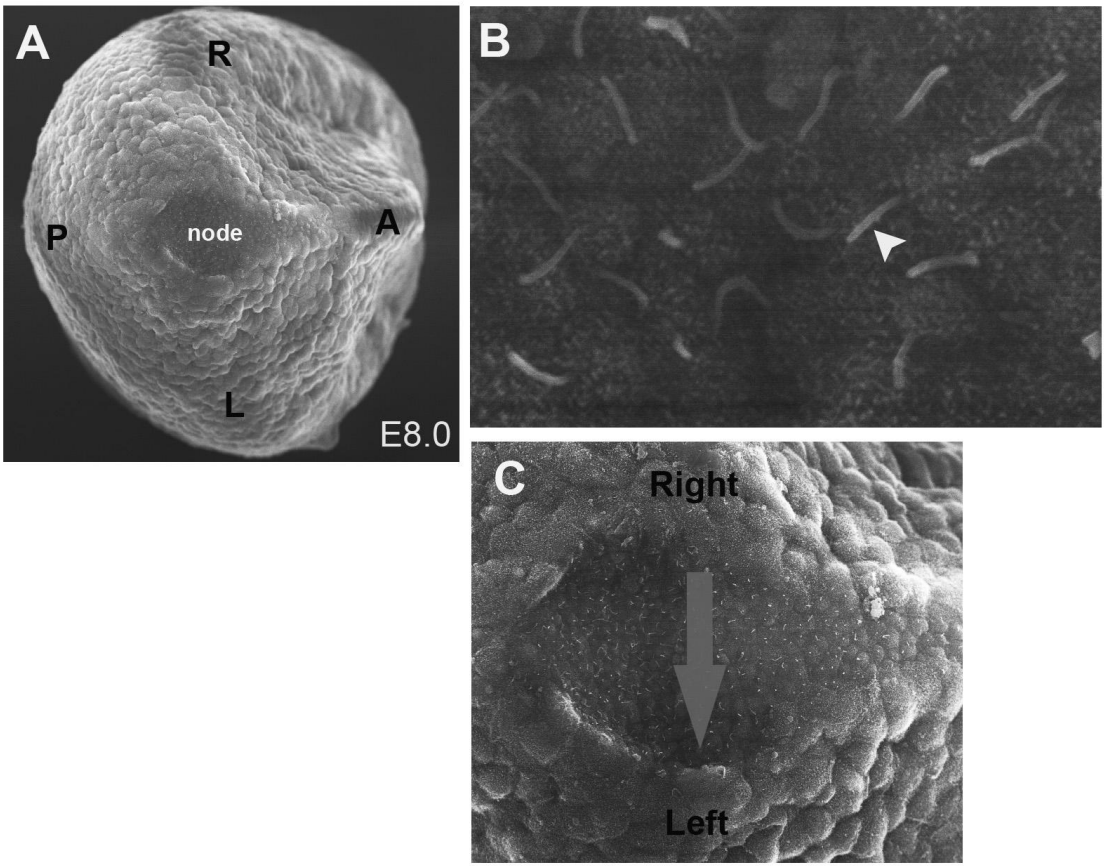


Figure 1.4 Node topology, cilia and nodal flow. The node is located in the distal part of the embryo (A) at E8.0, forming a depressed structure that is lined with projecting cilia (arrowhead in B) from each node cell. Cilia rotation causes the leftward flow (gray arrow in C) of extracellular fluid to the edge of the node on the left side, termed as nodal flow.

essential for determination of the LR axis.

To explain how nodal flow in the node functions at the signaling level, two models have been proposed: 1) The **morphogen model** proposes physical transport of lipophilic granules, called nodal vesicular parcels (NVPs), containing Sonic Hedgehog (Shh) and Retinoic acid (RA) molecules that are released upon contact with the left margin of the node in an FGF-dependent process (Tanaka et al., 2005), and 2) The **two-**

cilia model, in which leftward fluid flow is sensed by immotile mechanosensing cilia containing calcium-ion channel Polycystin2 (PKD2) but are devoid of left-right dynein, results in elevation of intracellular Ca^{2+} levels in the endoderm at the left margin of the node (McGrath et al., 2003). Recent studies from the Hamada group have shown that PKD2 localization on perinode cilia is required for mechanosensory input from nodal flow, providing support for the two-cilia model (Yoshiba et al., 2012). These models are based on expression patterns in the node and loss of function phenotypes; however, the roles of individual components in the models have not been rigorously tested so far.

Transfer of left-right signal from the node to the lateral plate mesoderm (LPM)

The asymmetric LR signal generated in the node has to be transported to the LPM, where it initiates expression of the TGFβ ligand, NODAL. It is still unknown how this signal transfers to the LPM. Among the possible routes of transfer of the signal from the node are: 1) definitive endoderm that lies on the exterior side, 2) paraxial mesoderm layer, and 3) the dorsal neuroectoderm layer (Figure 1.5A, B). Moreover, the nature of the signal that travels to the LPM is also unknown. While, it is known that *Nodal* expressed in the node is required for *Nodal* expression in the LPM, whether the transfer is direct or indirect through a secondary signal needs further investigation (Brennan et al., 2002; Saijoh et al., 2003). Significantly, it was found that there were elevated levels of free intracellular Ca^{2+}, specifically in the definitive endoderm cells lying between the node and the LPM on the left side (McGrath et al., 2003; Tabin and Vogan, 2003; Tanaka et al., 2005). This is the first (and only) reported sign of LR asymmetry subsequent to the establishment of nodal flow in the node and prior to the initiation of LR asymmetric gene

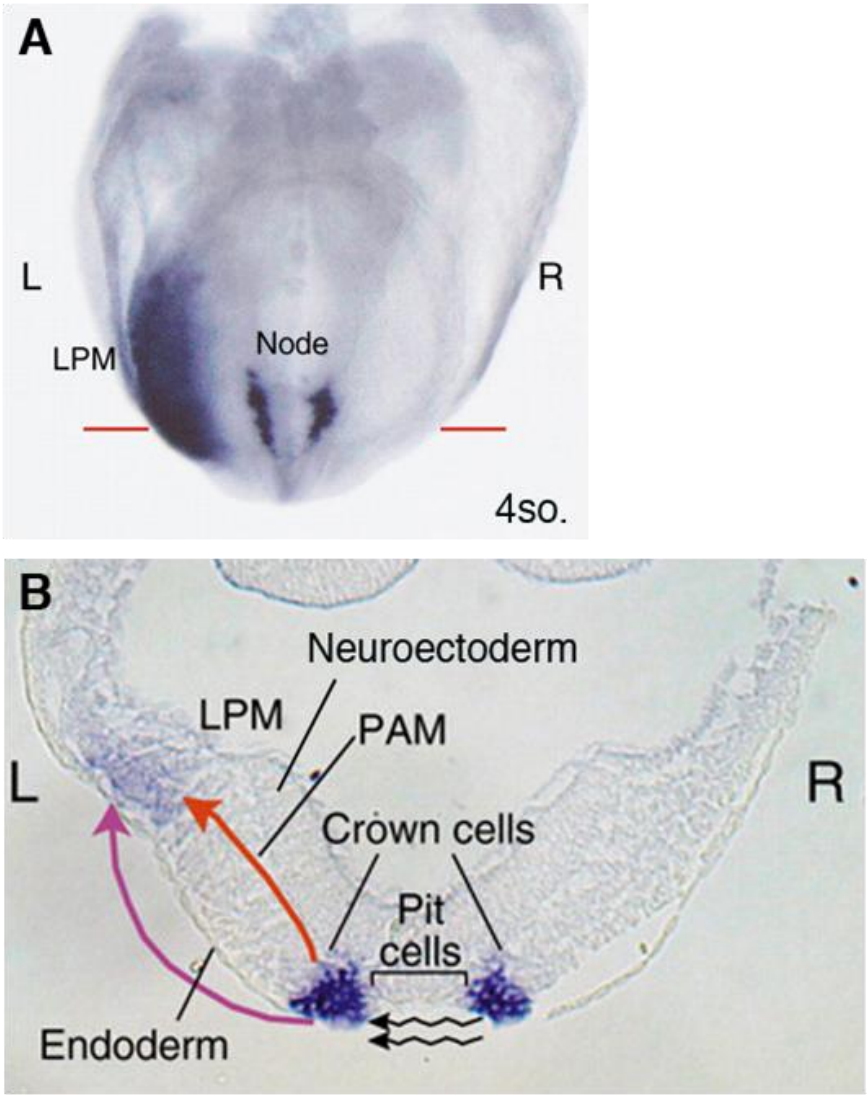


Figure 1.5 Modes of signal transfer from the node to the lateral plate mesoderm. (A) Transverse section of 4-somite stage embryo (*Nodal* in situ hybridization) across the node and the lateral plate mesoderm (LPM). *Nodal* expression in the LPM and the node reveals possible routes of LR signal from the node to the LPM (B) (Shiratori and Hamada, 2006): 1) through endoderm layer (purple arrow) that adjoins the node and overlies the LPM, 2) PAM (paraxial mesoderm, red arrow) lying between the endoderm and the neuroectoderm.

expression in the LPM. This suggests a potential role for free intracellular Ca^{2+} in the propagation of LR signal from the node to the LPM. However, more research is needed to establish whether flow of free Ca^{2+} through endoderm cells is necessary and sufficient for the signal transfer to the LPM. These studies will not only highlight the importance of definitive endoderm in LR determination, but also establish a central role for free Ca^{2+} in the transfer mechanism from the node to the LPM. Inhibition of biosynthesis of sulfated glycosaminoglycans (GAGs), heparan and chondriotin sulfate in cultured wildtype embryos leads to absence of *Nodal* expression, suggesting a role in the transfer of the asymmetric signal to the LPM (Oki et al., 2007). The sulfated GAGs are primarily localized to the basement membrane of the endoderm layer and were shown to physically interact with NODAL in *in vitro* conditions. The importance of NODAL produced in the node in LR signal transfer has been elegantly demonstrated by the use of a hypomorphic allele of *Nodal* that abolishes *Nodal* expression in the node and the LPM and the subsequent rescue of *Nodal* expression in the LPM by a transgene that expresses *Nodal* only in the perinode (also known as crown cells) (Saijoh et al., 2003). The perinode cells also show active Nodal signaling on the left side, the orientation of which is dependent on directional nodal flow (Kawasumi et al., 2011). Some perinode cells are found underneath the endoderm layer at the edge of the node, whereas the remaining perinode cells are in the periphery of the node. These data suggest that the perinode cells expressing *Nodal* are critical for LR determination and may require close interactions with the endoderm cells for the transmission of the asymmetric signal to the LPM.

Asymmetric gene expression in the lateral plate mesoderm

The expression of *Nodal* in the left LPM is critical for the asymmetric development of the visceral organs. At the 3-somite stage, *Nodal* expression in the left LPM begins near the node and expands to cover the entire LPM from the 4- to 6-somite stage and is undetectable at the 7-somite stage. The NODAL activity in the LPM is regulated by both positive and negative feedback loops in the Nodal signaling pathway that directly regulates expression of *Nodal* itself and the NODAL inhibitor LEFTY2, another secreted TGFβ ligand (Figure 1.6A; Adachi et al., 1999). Both *Nodal* and *Lefty2* expression in the LPM is regulated by the NODAL responsive enhancer ASE (Figure 1.6B; Adachi et al., 1999; Norris et al., 2002; Saijoh et al., 2000; Saijoh et al., 2005). Although asymmetric gene expression is seen in the left LPM, the right LPM is equally responsive to the NODAL signal as evidenced by mutants such as *iv*, where randomized LR determination causes bilateral, absent, right or left-sided gene expression (Collignon et al., 1996; Saijoh et al., 1996). This left and right *Nodal* responsiveness is due to the bilateral expression pattern of downstream Nodal signaling molecules in the LPM, such as *FoxH1*, *Cryptic* and *GDF1*, hence the initiating NODAL signal localized in a small region of the LPM causes activation and propagation of the Nodal signaling pathway to cover the entire LPM. This has been conclusively demonstrated by introduction of a *Nodal* expression vector into the precursors of the right LPM, which induces endogenous *Nodal* expression in the entire right LPM (Nakamura et al., 2006). Currently, the source of initial induction of Nodal signaling in the LPM in the developing embryo is unknown.

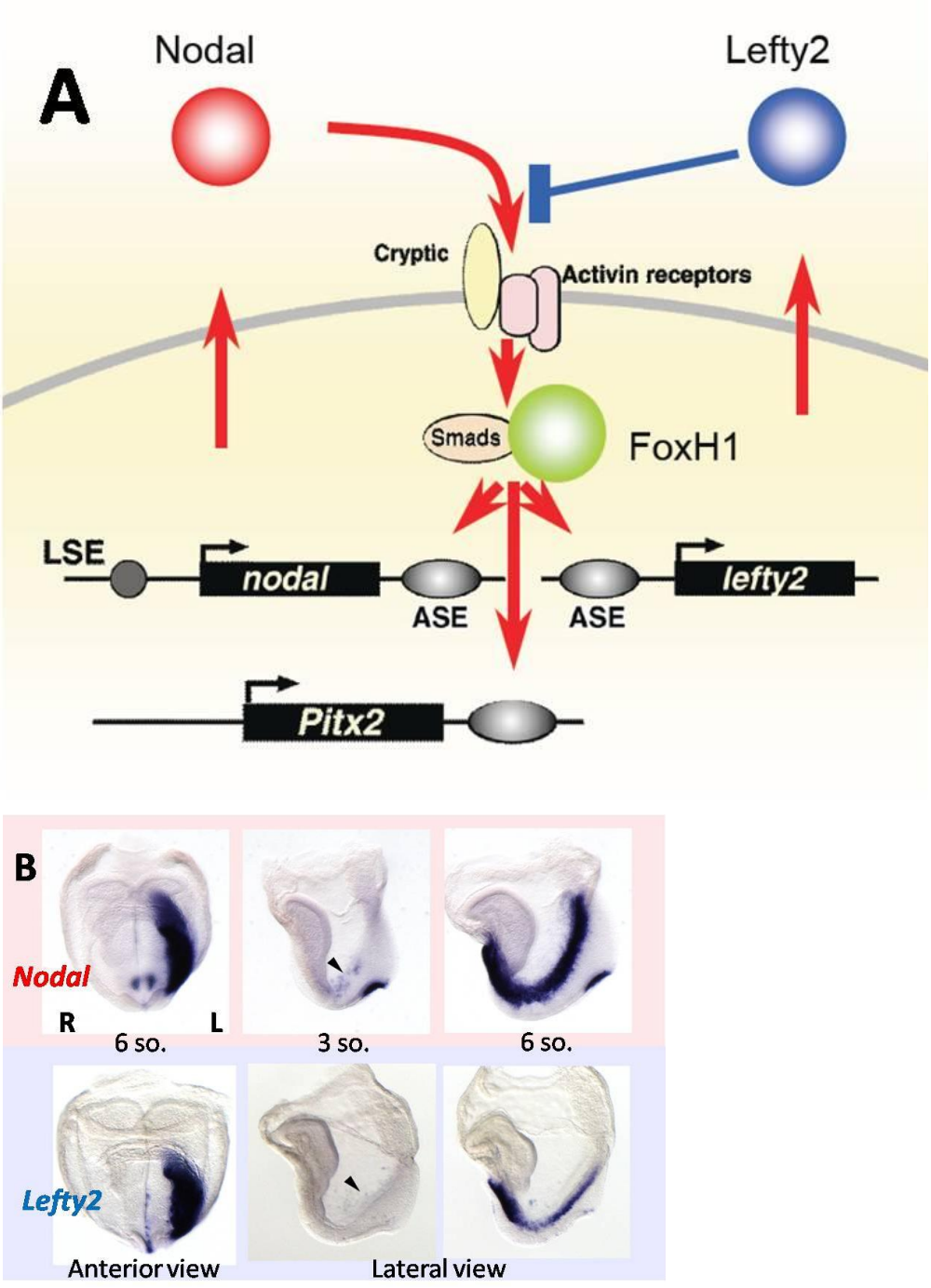


Figure 1.6 Activation of Nodal signaling pathway in the LPM. (A) The schematic representation of the Nodal signaling pathway, depicting the positive and negative feedback loops created by activation of *Nodal* and the secreted NODAL inhibitor, *Lefty2*. (B) Both *Nodal* and *Lefty2* are expressed in the left LPM at the 6-somite stage. Expression of both *Nodal* and *Lefty2* begins at the 3-somite stage (arrowheads) in the LPM near the node and reaches the maximum at 6-somite stage (Shiratori and Hamada, 2006).

Rationale and hypothesis

A fundamental question in the field of LR determination is the elucidation of the mechanisms involved in the transfer of the asymmetric signal from the node to the LPM. **I hypothesized that definitive endoderm plays a role in node morphogenesis and transfer of the LR signal from the node to the left LPM.** The following rationale supports the involvement of definitive endoderm in the transfer of LR signal from the node to the LPM in mouse embryos.

Common precursors of definitive endoderm and the node in the anterior primitive streak (APS)

At the primitive streak, ingression of epiblast cells undergoing an epithelial-mesenchymal transition causes the emergence of the definitive endoderm cells, which migrate outwards from the primitive streak (Tam et al., 2001). As the primitive streak elongates from E6.5-7.0, new definitive endoderm cells arise, especially from the anterior-most portion of the primitive streak, the anterior primitive streak (APS) (Lewis and Tam, 2006). These new definitive endoderm cells then migrate distally and anteriorly to cover the entire embryonic region of the embryo at the E7.5 stage. From E7.0-7.5, endoderm specific genes such as *Sox17*, *Foxa2*, *Cerberus1* and *Hex* are expressed in specific subsets of the entire definitive endoderm (Tam et al., 2003; Tam et al., 2007). In addition to becoming a source of definitive endoderm cells, the APS itself develops into the node at the distal end of the embryo (Lu et al., 2001). During this process, APS cells undergo differentiation into premature node cells with short cilia, while remaining covered with definitive endoderm cells (E7.5) (Figure 1.7A). The displacement of definitive endoderm overlying the differentiating node cells occurs from the E7.75-8.0

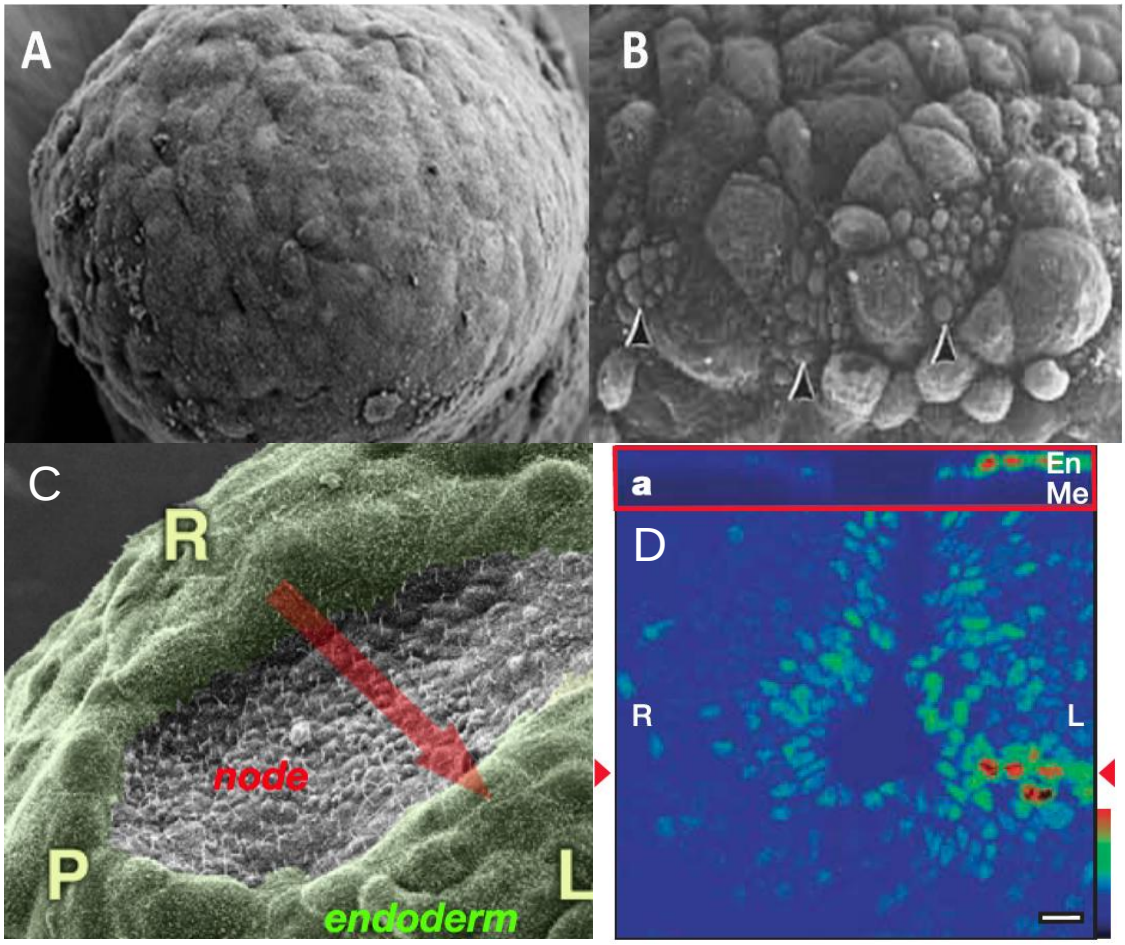


Figure 1.7 Endoderm cells may be important for node development and receiving left-right asymmetric signal from the node. (A) The distal-side view of an embryo at the bud stage, E7.5. The node precursors at this stage are covered with definitive endoderm cells and are not visible from the outside. (B) High magnification distal view of an embryo at E7.75, the definitive endoderm cells begin to move aside, exposing pockets of node cells (arrowheads) (Lee et al., 2008 and Sulik et al., 1994). (C) The endoderm layer (pseudocolor green) is positioned adjoining the node that generates the leftward nodal flow (red arrow) triggering the activation of the asymmetric signal on the left side (Shiratori and Hamada, 2006). (D) Higher levels of free intracellular Ca^{2+} in the endoderm cells (inset a) are seen on the left side of the node at the 3-somite stage, indicated by Fluo-3 dye signal (red – higher signal) (Tanaka et al., 2005).

and exposes these node cells to the extracellular environment, forming a depressed node structure (Figure 1.7B) (Lee and Anderson, 2008; Sulik et al., 1994). Thus, both definitive endoderm and node precursors remain in direct contact with each other during node morphogenesis from the APS (Figure 1.7C).

Foxa2 is expressed in the APS during the streak stages and in both the mature node and endoderm cells (Ang et al., 1993). The *Foxa2* null mutant embryos fail to form the node and also show severe foregut endoderm defects, suggesting a common origin of the node and the endoderm in the APS (Ang et al., 1994). In the mature node, the peripherally located perinode cells expressing *Nodal* continue to exist in close interaction with the overlying endoderm layer and may signal inductively with each other during different stages of LR determination (see Chapter 3). Thus, endoderm differentiation may impact node development and hence cause LR defects.

Endoderm may be involved in left-right signal transfer to the lateral plate mesoderm

The endoderm cells form the outermost layer of the embryo and remain contiguous with the node from the headfold to the early somite stages, when the nodal flow occurs (Figure 1.7C). Endoderm may be important for LR determination after node formation, as elevated levels of free Ca^{2+} are observed in definitive endoderm cells, specifically on the left side subsequent to nodal flow (Figure 1.7D; McGrath et al., 2003; Tanaka et al., 2005). This interesting finding suggests that endoderm cells may be involved in receiving the signaling output from nodal flow, since mutants such as *iv/iv* that lack directional nodal flow also lack asymmetric elevation of Ca^{2+} levels in endoderm (Tanaka et al., 2005). The activation of free intracellular Ca^{2+} on the left side

has also been observed in zebrafish and chicks subsequent to symmetry breaking at the node structure; however, it is not clear whether the activation occurs in the endoderm layer (Raya et al., 2004; Sarmah et al., 2005). It is conceivable that the Ca^{2+} signal in the endoderm cells is relayed from the node to the LPM where it activates asymmetric gene expression, although this possibility has not been directly tested. Together, these two pieces of evidence provided the basis to investigate the effects of endoderm on LR determination.

Sox17 mutants are an ideal candidate to study the role of endoderm in left-right determination. The known regulators of endoderm differentiation, HMG-box transcription factor *Sox17* and Forkhead-box family member *Foxa2*, show expression patterns and mutant phenotypes that suggest their possible involvement in generation of LR asymmetry. In zebrafish, *sox17* and *sox32* (*casanova*) are expressed both in endoderm cells and the dorsal forerunner cells, that are precursors of Kupffer's vesicle (Essner et al., 2005). Kupffer's vesicle is a closed ciliated organ analogous to the mouse node, where the initial symmetry breaking step takes place. In *casanova* mutants, apart from the loss of endoderm cells, the Kupffer's vesicle also does not form and LR defects occur (Alexander and Stainier, 1999).

In the mice, *Sox17* is the only *Sox* gene expressed in definitive endoderm cells but is not expressed in the node. *Sox17* shows a dynamic expression pattern in definitive and visceral endoderm from the streak during gastrulation and was reported to be expressed in the APS (Kanai-Azuma et al., 2002). In this study, the authors showed that definitive endoderm was formed but reduced in population in *Sox17* null mutant embryos. In another study, *Sox17* mutants were also shown to exhibit abnormal heart looping

suggesting LR defects (Sakamoto et al., 2007). The presence of the node and the partial endoderm defect in *Sox17* mutants (Kanai-Azuma et al., 2002) make *Sox17* an ideal candidate to study how definitive endoderm may play a role in all steps of LR determination.

Research summary

My dissertation research focusses on the role of endoderm in LR determination, by examination of the different steps of LR asymmetry and endoderm differentiation in *Sox17* mutants.

In the first part, we explore the expression pattern of *Sox17*, the affected endoderm genes in the mutants and the initial signs of LR asymmetry in the *Sox17* mutants (Chapter 2). Here, we showed that *Sox17* was the only endoderm regulator gene that was expressed in definitive endoderm between the node and the lateral plate mesoderm. The mutant embryos showed reduced expression of foregut markers, a severe loss of hindgut gene expression and complete downregulation of *Nephrocan*. Heart morphology in the mutant indicated a shortened tube length and revealed randomized looping orientation suggesting defects in LR determination. Asymmetric expression of *Nodal* and downstream targets, *Lefty2* and *Pitx2* was either absent or reduced in the left LPM in the *Sox17* mutants.

In the next section (Chapter 3), we investigate the three individual steps of LR determination in the *Sox17* mutants; breaking of asymmetry in the node, signal transfer to the LPM and the expansion of asymmetric gene expression in the LPM. We showed that nodal flow and LR asymmetry across the node occurred in the normal orientation in the

mutant, and the LPM was capable of activating endogenous *Nodal* expression when supplied with an external source of NODAL. Connexin43, the major gap junction protein was found to be absent from the endoderm in *Sox17* mutants and gap junction permeability was severely compromised in the mutant endoderm cells. The epithelial morphology in the mutants was markedly abnormal with disorganized adhesion junction complexes between endoderm cells and mesenchymal like cellular morphology. The other tissues, such as paraxial mesoderm and midline, were found to be relatively normal as indicated by proteoglycan distribution and marker gene expression. These data clearly suggested that defects in endoderm differentiation resulted in a loss of LR signal information from the node to the LPM in the *Sox17* mutants.

We then further studied the endoderm differentiation in mutant embryos by examining the regulatory mechanisms that resulted in the prolonged expression of *Nodal*, an upstream regulator of endoderm differentiation in the primitive streak, in the foregut endoderm in *Sox17* mutants (Chapter 4). In normal embryos, *Nodal* expression in the definitive endoderm is derived from the primitive streak and ceases at the early bud stages to allow differentiation to occur. In *Sox17* mutants, the endoderm specific enhancer of *Nodal* was found to be continuously expressed, leading to activation of the Nodal signaling pathway in the foregut endoderm at the early somite stages. Conditional deletion of *Sox17* at the poststreak stages led to a reduction of ectopic *Nodal* expression in the foregut. This suggests that downregulation of *Nodal* expression in endoderm depends on the onset of *Sox17* expression in the definitive endoderm during the streak stages.

References

Adachi, H., Saijoh, Y., Mochida, K., Ohishi, S., Hashiguchi, H., Hirao, A. and Hamada, H. (1999). Determination of left/right asymmetric expression of nodal by a left side-specific enhancer with sequence similarity to a lefty-2 enhancer. *Genes Dev* **13**(12), 1589-600.

Alexander, J. and Stainier, D. Y. (1999). A molecular pathway leading to endoderm formation in zebrafish. *Curr Biol* **9**(20), 1147-57.

Ang, S. L., Wierda, A., Wong, D., Stevens, K. A., Cascio, S., Rossant, J. and Zaret, K. S. (1993). The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins. *Development* **119**(4), 1301-15.

Ang, S. L. and Rossant, J. (1994). HNF-3 beta is essential for node and notochord formation in mouse development. *Cell* **78**(4), 561-74.

Brennan, J., Norris, D. P. and Robertson, E. J. (2002). Nodal activity in the node governs left-right asymmetry. *Genes Dev* **16**(18), 2339-44.

Capdevila, J., Vogan, K. J., Tabin, C. J. and Izpisua Belmonte, J. C. (2000). Mechanisms of left-right determination in vertebrates. *Cell* **101**(1), 9-21.

Dufort, D., Schwartz, L., Harpal, K. and Rossant, J. (1998). The transcription factor HNF3beta is required in visceral endoderm for normal primitive streak morphogenesis. *Development* **125**(16), 3015-25.

Essner, J. J., Amack, J. D., Nyholm, M. K., Harris, E. B. and Yost, H. J. (2005). Kupffer's vesicle is a ciliated organ of asymmetry in the zebrafish embryo that initiates left-right development of the brain, heart and gut. *Development* **132**(6), 1247-60.

Hamada, H., Meno, C., Watanabe, D. and Saijoh, Y. (2002). Establishment of vertebrate left-right asymmetry. *Nat Rev Genet* **3**(2), 103-13.

Hirokawa, N., Tanaka, Y., Okada, Y. and Takeda, S. (2006). Nodal flow and the generation of left-right asymmetry. *Cell* **125**(1), 33-45.

Kaestner, K. H., Hiemisch, H., Luckow, B. and Schutz, G. (1994). The HNF-3 gene family of transcription factors in mice: gene structure, cDNA sequence, and mRNA distribution. *Genomics* **20**(3), 377-85.

Kanai-Azuma, M., Kanai, Y., Gad, J. M., Tajima, Y., Taya, C., Kurohmaru, M., Sanai, Y., Yonekawa, H., Yazaki, K., Tam, P. P. et al. (2002). Depletion of definitive gut endoderm in Sox17-null mutant mice. *Development* **129**(10), 2367-79.

Kathiriya, I. S. and Srivastava, D. (2000). Left-right asymmetry and cardiac looping: implications for cardiac development and congenital heart disease. *Am J Med Genet* **97**(4), 271-9.

Kawasumi, A., Nakamura, T., Iwai, N., Yashiro, K., Saijoh, Y., Belo, J. A., Shiratori, H. and Hamada, H. (2011). Left-right asymmetry in the level of active Nodal protein produced in the node is translated into left-right asymmetry in the lateral plate of mouse embryos. *Dev Biol* **353**(2), 321-30.

Kosaki, K. and Casey, B. (1998). Genetics of human left-right axis malformations. *Semin Cell Dev Biol* **9**(1), 89-99.

Lee, J. D. and Anderson, K. V. (2008). Morphogenesis of the node and notochord: the cellular basis for the establishment and maintenance of left-right asymmetry in the mouse. *Dev Dyn*. **237**(12), 3464-76.

Lee, J. D., Migeotte, I. and Anderson, K. V. (2010). Left-right patterning in the mouse requires Epb4.1l5-dependent morphogenesis of the node and midline. *Dev Biol* **346**(2), 237-46.

Lewis, S. L. and Tam, P. P. (2006). Definitive endoderm of the mouse embryo: formation, cell fates, and morphogenetic function. *Dev Dyn* **235**(9), 2315-29.

Lu, C. C., Brennan, J. and Robertson, E. J. (2001). From fertilization to gastrulation: axis formation in the mouse embryo. *Curr Opin Genet Dev* **11**(4), 384-92.

McGrath, J., Somlo, S., Makova, S., Tian, X. and Brueckner, M. (2003). Two populations of node monocilia initiate left-right asymmetry in the mouse. *Cell* **114**(1), 61-73.

Nakamura, T., Mine, N., Nakaguchi, E., Mochizuki, A., Yamamoto, M., Yashiro, K., Meno, C. and Hamada, H. (2006). Generation of robust left-right asymmetry in the mouse embryo requires a self-enhancement and lateral-inhibition system. *Dev Cell* **11**(4), 495-504.

Nonaka, S., Shiratori, H., Saijoh, Y. and Hamada, H. (2002). Determination of left-right patterning of the mouse embryo by artificial nodal flow. *Nature* **418**(6893), 96-9.

Nonaka, S., Tanaka, Y., Okada, Y., Takeda, S., Harada, A., Kanai, Y., Kido, M. and Hirokawa, N. (1998). Randomization of left-right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. *Cell* **95**(6), 829-37.

Nonaka, S., Yoshiba, S., Watanabe, D., Ikeuchi, S., Goto, T., Marshall, W. F. and Hamada, H. (2005). De novo formation of left-right asymmetry by posterior tilt of nodal cilia. *PLoS Biol* **3**(8), e268.

Okada, Y., Nonaka, S., Tanaka, Y., Saijoh, Y., Hamada, H. and Hirokawa, N. (1999). Abnormal nodal flow precedes situs inversus in iv and inv mice. *Mol Cell* **4**(4), 459-68.

Okada, Y., Takeda, S., Tanaka, Y., Izpisua Belmonte, J. C. and Hirokawa, N. (2005). Mechanism of nodal flow: a conserved symmetry breaking event in left-right axis determination. *Cell* **121**(4), 633-44.

Oki, S., Hashimoto, R., Okui, Y., Shen, M. M., Mekada, E., Otani, H., Saijoh, Y. and Hamada, H. (2007). Sulfated glycosaminoglycans are necessary for Nodal signal transmission from the node to the left lateral plate in the mouse embryo. *Development* **134**(21), 3893-904.

Peeters, H. and Devriendt, K. (2006). Human laterality disorders. *Eur J Med Genet* **49**(5), 349-62.

Raya, A., Kawakami, Y., Rodriguez-Esteban, C., Ibanes, M., Rasskin-Gutman, D., Rodriguez-Leon, J., Buscher, D., Feijo, J. A. and Izpisua Belmonte, J. C. (2004). Notch activity acts as a sensor for extracellular calcium during vertebrate left-right determination. *Nature* **427**(6970), 121-8.

Robb, L. and Tam, P. P. (2004). Gastrula organiser and embryonic patterning in the mouse. *Semin Cell Dev Biol* **15**(5), 543-54.

Saijoh, Y., Adachi, H., Sakuma, R., Yeo, C. Y., Yashiro, K., Watanabe, M., Hashiguchi, H., Mochida, K., Ohishi, S., Kawabata, M. et al. (2000). Left-right asymmetric expression of lefty2 and nodal is induced by a signaling pathway that includes the transcription factor FAST2. *Mol Cell* **5**(1), 35-47.

Meno, C., Saijoh, Y., Fujii, H., Ikeda, M., Yokoyama, T., Yokoyama, M., Toyoda, Y. and Hamada, H. (1996). Left-right asymmetric expression of the TGF beta-family member lefty in mouse embryos. *Nature* **381**(6578), 151-5.

Saijoh, Y., Oki, S., Ohishi, S. and Hamada, H. (2003). Left-right patterning of the mouse lateral plate requires nodal produced in the node. *Dev Biol* **256**(1), 160-72.

Saijoh, Y., Oki, S., Tanaka, C., Nakamura, T., Adachi, H., Yan, Y. T., Shen, M. M. and Hamada, H. (2005). Two nodal-responsive enhancers control left-right asymmetric expression of Nodal. *Dev Dyn* **232**(4), 1031-6.

Sakamoto, Y., Hara, K., Kanai-Azuma, M., Matsui, T., Miura, Y., Tsunekawa, N., Kurohmaru, M., Saijoh, Y., Koopman, P. and Kanai, Y. (2007). Redundant roles of Sox17 and Sox18 in early cardiovascular development of mouse embryos. *Biochem Biophys Res Commun* **360**(3), 539-44.

Sarmah, B., Latimer, A. J., Appel, B. and Wente, S. R. (2005). Inositol polyphosphates regulate zebrafish left-right asymmetry. *Dev Cell* **9**(1), 133-45.

Sulik, K., Dehart, D. B., Iangaki, T., Carson, J. L., Vrablic, T., Gesteland, K. and Schoenwolf, G. C. (1994). Morphogenesis of the murine node and notochordal plate. *Dev Dyn* **201**(3), 260-78.

Tabin, C. J. and Vogan, K. J. (2003). A two-cilia model for vertebrate left-right axis specification. *Genes Dev* **17**(1): 1-6.

Tam, P. P., Gad, J. M., Kinder, S. J., Tsang, T. E. and Behringer, R. R. (2001). Morphogenetic tissue movement and the establishment of body plan during development from blastocyst to gastrula in the mouse. *Bioessays* **23**(6), 508-17.

Tam, P. P., Kanai-Azuma, M. and Kanai, Y. (2003). Early endoderm development in vertebrates: lineage differentiation and morphogenetic function. *Curr Opin Genet Dev* **13**(4), 393-400.

Tam, P. P., Khoo, P. L., Lewis, S. L., Bildsoe, H., Wong, N., Tsang, T. E., Gad, J. M. and Robb, L. (2007). Sequential allocation and global pattern of movement of the definitive endoderm in the mouse embryo during gastrulation. *Development* **134**(2), 251-60.

Tanaka, Y., Okada, Y. and Hirokawa, N. (2005). FGF-induced vesicular release of Sonic hedgehog and retinoic acid in leftward nodal flow is critical for left-right determination. *Nature* **435**(7039), 172-7.

Yoshiba, S., Shiratori, H., Kuo, I. Y., Kawasumi, A., Shinohara, K., Nonaka, S., Asai, Y., Sasaki, G., Belo, J. A., Sasaki, H. et al. (2012). Cilia at the node of mouse embryos sense fluid flow for left-right determination via Pkd2. *Science* **338**(6104), 226-31.

CHAPTER 2

EARLY STUDIES ON *SOX17* EXPRESSION, ENDODERM DIFFERENTIATION AND LEFT-RIGHT DEFECTS

Introduction

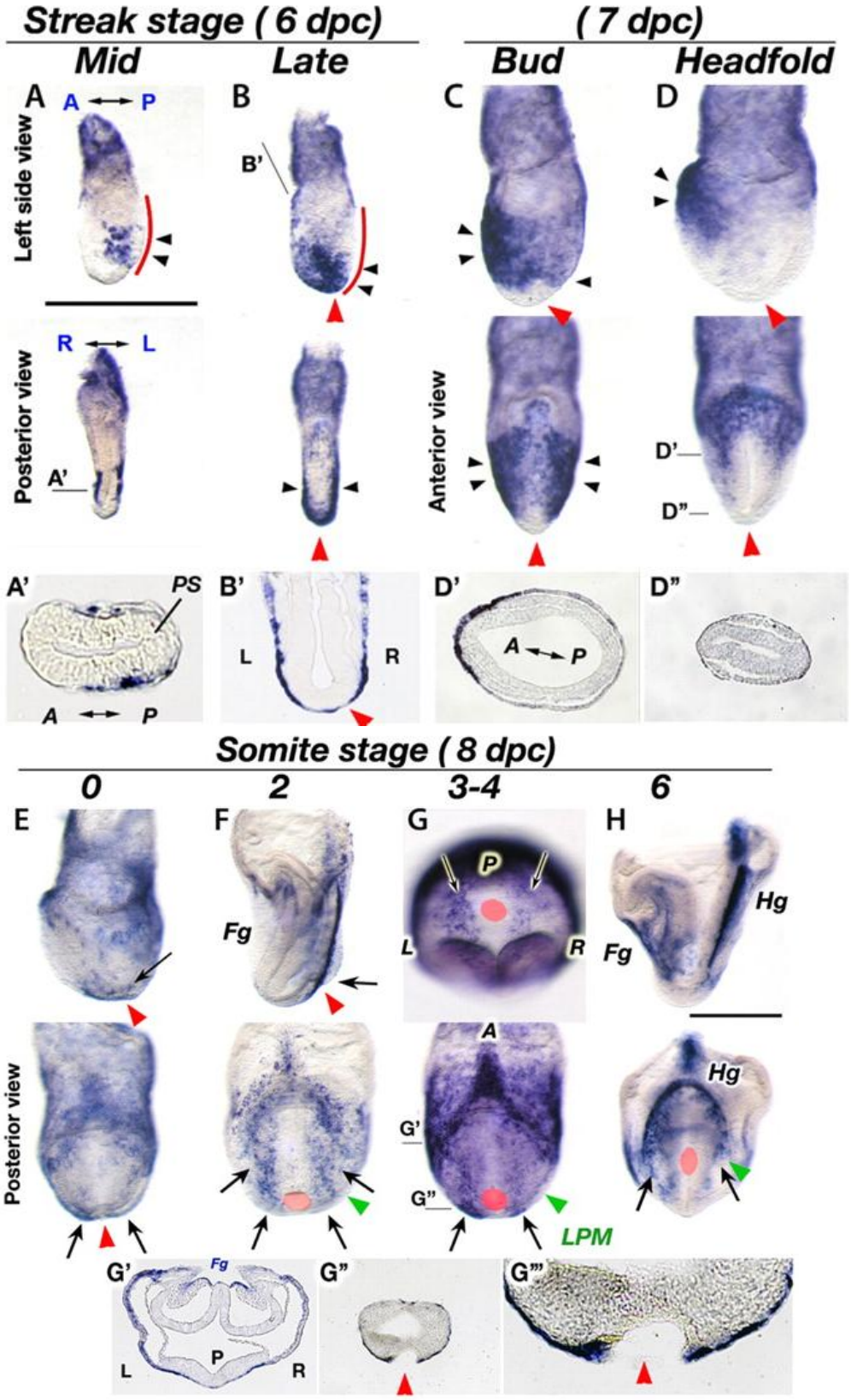
During gastrulation, definitive endoderm cells arise from the primitive streak and undergo dynamic migration patterns to localize at distinct regions of the gut tube. Although definitive endoderm cells give rise to most of the gut tube, recent studies have shown that a small proportion of extraembryonic endoderm derived cells are incorporated into the gut tube (Kwon et al., 2008). These extraembryonic endoderm cells exist amongst the definitive endoderm at the poststreak stages forming a single layer. This thesis research focuses on the role of the endoderm layer during the three phases of left-right (LR) determination; hence critical examination of the gene expression patterns of early endoderm regulators is required to understand how they may regulate or interact with cells and tissues involved in LR determination. Cell fate studies have shown that definitive endoderm precursors positioned at different regions of the poststreak embryo (E7.0) migrate to different final positions such as the foregut and the midgut tissues at E8.5 (Tam et al., 2007). It is also known that during the migratory phase from E7.0 to E8.5, subsets of the entire endoderm layer express different endoderm genes such as *Sox17*, *Foxa2*, *Cerberus* and *Hex*, which are examined further. Our expression pattern analysis has suggested that *Sox17* is the ideal candidate to examine the role of the

endoderm layer in the determination of LR asymmetry. We then characterized the *Sox17* mutant phenotypes to understand how SOX17 regulates endoderm differentiation in different parts of the embryonic gut with regards to LR determination.

Expression of *Sox17* in definitive endoderm

The HMG-box transcription factor, SOX17 is known as a master regulator of endoderm differentiation in vertebrates (Tam et al., 2003). Previously, expression patterns of *Sox17* have been shown for the midstreak (E6.75), midbud (E7.5) and headfold (E8.0) stages (Kanai-Azuma et al., 2002). Since the definitive endoderm migrates rapidly from the primitive streak (posterior region) to the anterior part of the embryo, the existing data do not sufficiently represent the dynamic expression profile with regards to development of LR asymmetry and how *Sox17* expressing cells may interact with other cell types, such as mesoderm and anterior primitive streak. Therefore, it is essential to examine expression in wildtype embryos at smaller stage intervals. Moreover, the published data do not clearly show the expression regions, due to low sensitivity and high background staining in the figures (Kanai-Azuma et al., 2002). To overcome these shortcomings, we have developed separate *Sox17* RNA probes against fragments over the entire length of the *Sox17* mRNA for greater sensitivity. We also optimized our *in situ* hybridization protocol for stringent RNA-probe binding to reduce background staining. These modifications have allowed detection of endoderm cells with low levels of *Sox17* expression with no background. We have analyzed *Sox17* expression from E6.5 (early streak) to E8.5 (5- to 6-somite stage) on several wildtype embryos at short stage intervals, forming a comprehensive expression profile to develop a better understanding of the role of *Sox17* in LR patterning during these stages (Figure 2.1).

Figure 2.1 Dynamic *Sox17* expression in definitive endoderm cells at stages when LR determination occurs. *Sox17* expression was initiated in migrating lateral endoderm cells at midstreak stage (A) and became expanded at the late streak stage (B) and the bud stage (C), then, the expression was refined to the anterior foregut region at the headfold stage (D). Black arrowheads represent *Sox17* expressing cells. Red lines indicate the primitive streak region. A new domain of *Sox17* expression is observed in the endoderm cells near the node from the 0-somite stage (E) to 4-somite stage (F, G, arrows). At E8.5, *Sox17* is prominently expressed in the foregut (Fg) and hindgut (Hg) pockets (H). A', B', D' and D", G'-G"' represent sections of A, B, D, and G, respectively, where positions of the sections are indicated with lines. Red arrowheads: position of the node precursors; red circles: position of the node; green arrowheads: lateral plate mesoderm (LPM). PS: primitive streak. Scale bars: 500 μm.


Streak stage (6 dpc)
(7 dpc)
Mid
Late
Bud
Headfold
A
A
P
Left side view
B
B'
C
D
R
L
Posterior view
A'
Anterior view
D'
D"
A'
PS
A
P
B'
L
R
D'
A
P
D"
Somite stage (8 dpc)
0
2
3-4
6
E
F
Fg
G
P
L
R
H
Hg
Fg
Posterior view
A
G'
G"
Hg
LPM
G'
Fg
L
P
R
G"
G'''

Sox17 expression overlies node precursors

From E6.5 to E7.0 (early to late streak stage), *Sox17* is expressed in the newly emerged definitive endoderm cells from the anterior end of the primitive streak, as the primitive streak extends to the distal region of the embryo (Figure 2.1A). At E7.0 (late streak), the *Sox17* expression in the definitive endoderm completely encompasses the entire distal region of the embryo that overlies the anterior primitive streak (APS) (Figure 2.1B). This expression pattern has important implications for endoderm dependence for node development since the cells in the APS that are in contact with the *Sox17* expressing definitive endoderm later form the mature node structure at the headfold stage (E8.0). It is also interesting to note that after the late streak stage (E7.0), *Sox17* ceases to be expressed in the distal region of the embryo, as the expression pattern expands to the anterior region of the embryo from early-bud (E7.25) to the head-fold stage (E8.0) (Figure 2.1C). At E7.5 (late bud), *Sox17* is broadly expressed in the entire definitive endoderm, except the distal region, that overlies node precursors. Thus, there is a narrow window of time (E7.0 to E7.25) when *Sox17* may influence node development in the anterior primitive streak.

Sox17 expression adjacent to the node

At the early headfold stage (E8.0), *Sox17* is expressed in the definitive endoderm in the anterior part of the embryo and there is no expression in the remaining embryo (Figure 2.1D). The node first appears at the late headfold stage (E8.0). At this stage, a new domain of *Sox17* expression is seen in the definitive endoderm cells immediately lateral to the node (Figure 2.1E). This regional expression of *Sox17* subsequently expands to form two bilateral stripes of definitive endoderm cells adjacent to the node and the

primitive streak on either side from 0- to 3-somite stages (Figure 2.1E-G). In a heterozygous *Sox17*$^{GFP/+}$ embryo where a GFP expression cassette replaces the *Sox17* coding region, the expression of GFP under the control of endogenous *Sox17* regulatory mechanism (Kim et al., 2007) is observed in most endoderm cells adjacent to the node at 3- to 4-somite stage (Figure 2.2A). Whole mount immunohistochemistry for SOX17 in the same embryo also reveals that the SOX17 is localized in the nucleus of the endoderm cells on either side of the node (Figure 2.2B). This expression pattern is highly interesting since it suggests that *Sox17* expressing definitive endoderm cells may be important for receiving signals arising from the node. The significance of this expression pattern is evident from previous studies that have shown elevated intracellular free Ca^{2+} levels in definitive endoderm cells adjacent to the node on the left side at the 2- to 3-somite stage (see Chapter 1, Figure 1.5C; McGrath et al., 2003; Tanaka et al., 2005). Elevated Ca^{2+} levels may be the signaling output from the node after breakage of symmetry at this stage, although it is not been critically examined. Thus, it is highly likely that there is

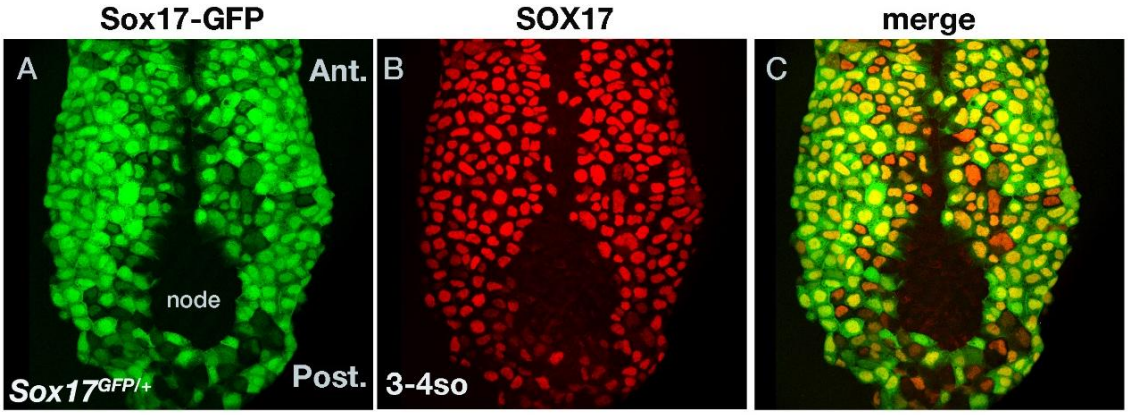


Figure 2.2 GFP and SOX17 protein localization in definitive endoderm near the node in a *Sox17*$^{GFP/+}$ embryo. (A) GFP is expressed both in the cytoplasm and the nucleus almost in the entire endoderm layer at 3- to 4-somite stage; (B) SOX17 protein is localized in the nucleus of endoderm cells; (C) merge of GFP and SOX17 protein expression.

overlap in *Sox17* expression and elevated Ca^{2+} levels seen in the definitive endoderm cells. This suggests that the function of SOX17 in these cells may be important for the initiation/maintenance of elevated Ca^{2+} levels to mediate transduction of LR signals from the node to the lateral plate mesoderm (LPM), following symmetry breaking by nodal flow.

Expression of other endoderm markers

FOXA2, also known as HNF3beta, is a forkhead transcription factor family member, predominantly expressed in the liver in the adult and required for the regulation of liver specific gene expression (Kaestner et al., 1994; Lee et al., 2005; Liu et al., 1991). *Foxa2* null mutants are embryonic lethal, lack a distinct node and the notochord and exhibit severe defects in foregut morphogenesis; however, the hindgut is relatively unaffected (Ang and Rossant, 1994). At the streak stages from E6.5 to E7.0, *Foxa2* is strongly expressed in the anterior primitive streak (APS) and in definitive endoderm neighboring the APS (Figure 2.3A). At the late bud stage (E7.5), *Foxa2* expression expands to encompass the entire definitive endoderm in the anterior part of the embryo that is later localized to the foregut pocket at the 4- to 5-somite stages at E8.5 (Figure 2.3B,C). *Foxa2* is essential for node formation and the midline structures. This suggests a role for *Foxa2* in the LR symmetry breaking event, supported by the absent expression of *Nodal* and bilateral or absent expression of *Lefty2* in the LPM in tetraploid *Foxa2* mutants. *Foxa2* function in tetraploid *Foxa2* mutants is specifically lost in the embryonic cells, whereas extraembryonic cells retain *Foxa2* function allowing the embryo to develop until E9.5 (Dufort et al., 1998). In our studies, we found that conditional deletion of *Foxa2* in the entire epiblast with *Sox2-cre* also results in the absence of the node and

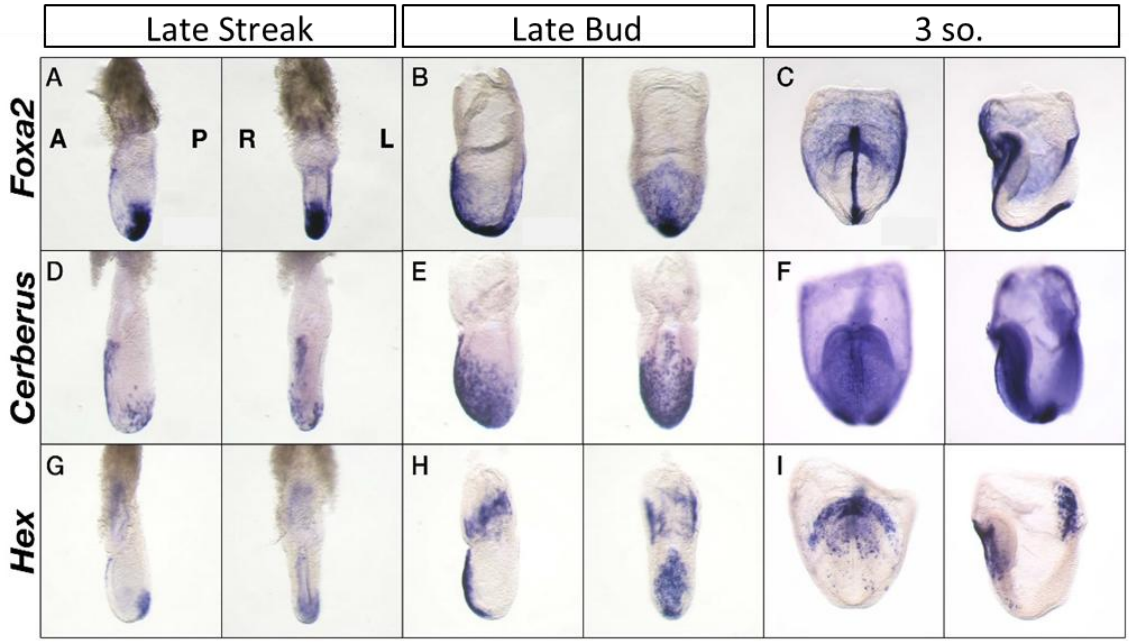


Figure 2.3 Expression patterns of *Foxa2*, *Cerberus* and *Hex* during early gastrulation stages reveal foregut localization at 3-somite stages. (A-C) *Foxa2* is expressed in the anterior primitive streak (APS) at the late streak stage (A), anterior definitive endoderm at bud stages (B) and in the foregut and at the midline at the 3-somite stage, but absent from posterior endoderm or near the node (C). (D-F) *Cerberus* is expressed in the distal definitive endoderm during late streak (D) and bud stages (E) and in the foregut and somites at the 3-somite stage (F). (G-I) *Hex* expression is seen in distal (G), anterior (H) definitive endoderm, and localized to the foregut at the 3-somite stage (I).

the midline (data not shown).

Cerberus (CER-1) is a secreted factor that is expressed in the anterior visceral endoderm (AVE) and functions as an antagonist of BMP and Wnt signaling (Biben et al., 1998; Piccolo et al., 1999). *Cer-1* is also expressed in the definitive endoderm originating from the APS at the streak stage and quickly expands to cover the anterior half of the embryo at the bud-stages (E7.5) (Figure 2.3D,E). At the early somite stages, *Cer-1* expression is restricted to the foregut and the newly formed somites and is absent from the hindgut (Figure 2.3F). *Cer-1* null mutants are normal, suggesting that other genes compensate for the loss of *Cer-1* activity for the specification of the different germ layers

(Belo et al., 2000).

Hex is a divergent homeobox gene transcription factor that is required for liver formation and plays an essential role in the hepatic endoderm progenitors (Martinez Barbera et al., 2000). During the early stages of gastrulation, *Hex* is strongly expressed in the AVE and the anterior definitive endoderm and becomes restricted to the ventral foregut and the allantois at the early somite stages (Figure 2.3G, H; Thomas et al., 1998). *Hex* is not expressed in the midgut or the posterior endoderm region adjacent to the developing node during the headfold to 4-somite stages (Figure 2.3I). *Hex* mutants survive until E10.5 and die due to impaired liver development and do not exhibit defects in other endoderm derived tissues (Martinez Barbera et al., 2000).

Gut endoderm defects in *Sox17* mutants

Our expression pattern analysis of *Sox17* at different stages of gastrulation showed strong expression in endoderm that forms the gut tube (Figure 2.1). Endoderm genes, *Foxa2* and *Hex* are expressed in the anterior definitive endoderm and become restricted to the foregut endoderm at the early somite stages (Figure 2.3). To address how endoderm differentiation is affected in *Sox17* mutants, we performed whole mount *in situ* hybridization for *Foxa2* and *Hex* at presomite and early somite stages. *Foxa2* and *Hex* expression were reduced in the foregut, suggesting a depletion of foregut endoderm in *Sox17* mutants especially in the lateral and ventral region of the foregut (Figure 2.4A-D). This observation correlates well with the abnormal foregut morphology seen in the *Sox17* mutant embryos. The hindgut expression of *Foxa2* is more severely affected in the *Sox17* mutants, suggesting an important role for SOX17 in the specification of the hindgut

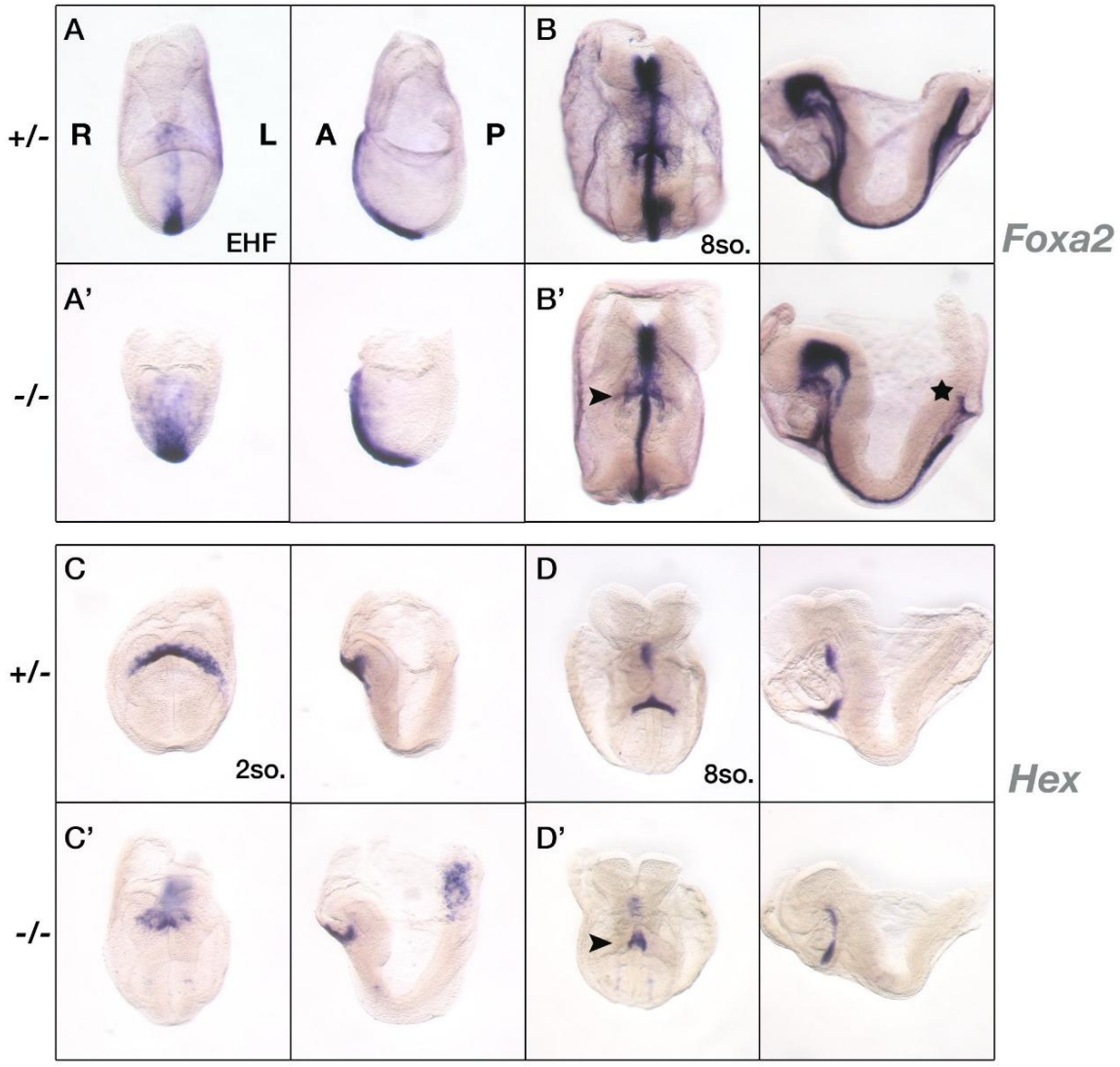


Figure 2.4 Foregut and hindgut defects in *Sox17* mutants. (A-B') *Foxa2* is expressed in the anterior endoderm at the early headfold stage (A) and in the foregut and hindgut endoderm at the 7- to 8-somite stage (B). In *Sox17* mutants, *Foxa2* expression is reduced in the foregut (B', arrow) and severely downregulated in the hindgut (B', star). (C-D') *Hex* expression is observed in the foregut at the 2-somite stage (C) and 7- to 8-somite stage (D). *Sox17* mutants show reduced expression of *Hex* in the lateral foregut region (D', arrow).

endoderm (Figure 2.4B'). Similar defects in hindgut expression of *Sonic Hedgehog* (*Shh*) were also observed in the *Sox17* mutants at the 7- to 8-somite stages. The hindgut is derived from the progenitor endoderm population that corresponds to the posterior region of the node at the early somite stages (Franklin et al., 2008). Thus, *Sox17* is the primary endoderm regulator gene that plays a critical role in the expansion and patterning of definitive endoderm in the posterior part of the embryo during early development.

Loss of *Nepn* expression in the midgut endoderm

Cell labeling studies have shown that the midgut region of the embryonic gut tube derives from the endoderm at the level of the node during the early somite stages (Franklin et al., 2008; Wilson and Beddington, 1996). *Nephrocan* (*Nepn*) was reported to be specifically expressed in the midgut endoderm region from the 4-somite stage onwards and in the gut tube at E9.5 (Hou et al., 2007). We found in *Sox17* mutants that *Nepn* expression was completely abrogated at the 7-somite stage and severely reduced at E9.5 in all embryos that were examined (n=9), compared to *Sox17* heterozygous control embryos (n=8) (Figure 2.5). In collaboration with Dr. Pamela Hoodless at the University of British Columbia, we have shown that *Nepn* may be a direct downstream target of *Sox17* (manuscript under preparation). Loss of *Nepn* expression in the endoderm neighboring the node suggests that these endoderm cells are in an incomplete state of differentiation in the *Sox17* mutants (Figure 2.5A, B).

This is a significant finding since complete downregulation of a target gene has been observed in *Sox17* mutants *in vivo* for the first time. These results also suggest that the bilateral expression of *Sox17* in the endoderm adjoining the node plays a role in endoderm differentiation, thereby meriting further analysis of cellular and morphological

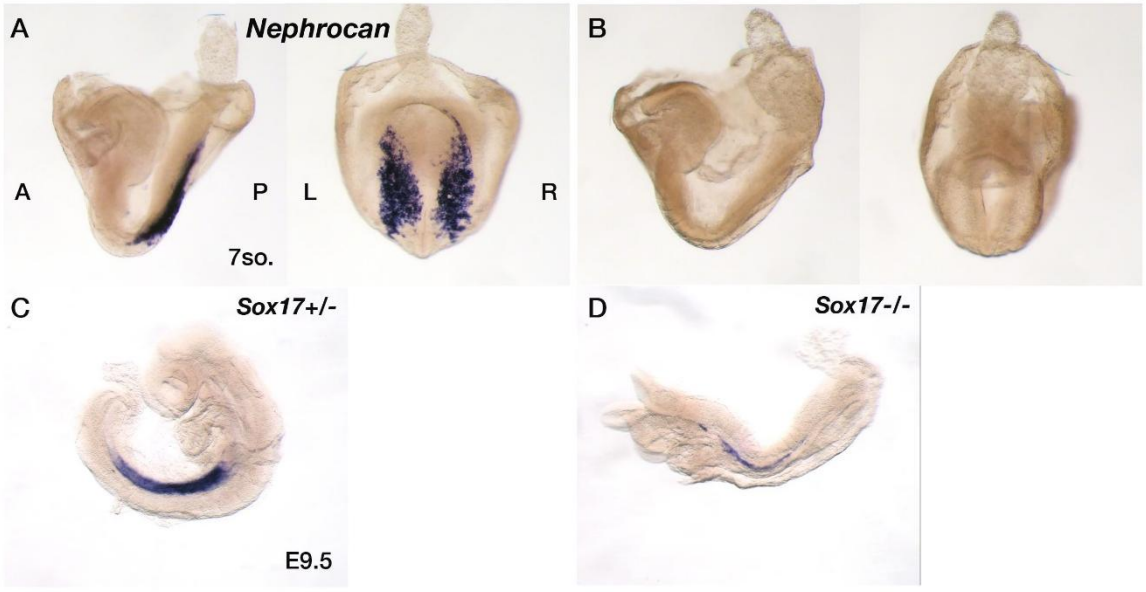


Figure 2.5 *Nephrocan* (*Nepn*) expression in the midgut region adjoining the node is downregulated in the *Sox17* mutants. (A, B) *Nepn* expression in the control (A) and *Sox17* mutant embryo at 7-somite stage. (C, D) At E9.5, *Nepn* is expressed in the midgut tube in the control embryo and is severely reduced in the *Sox17* mutant embryo.

abnormalities of the endoderm in this region in the mutants (Chapter 3). *Nepn* expression begins at the 4-somite stage in normal embryos and was found to be absent at this stage in *Sox17* mutants (data not shown), indicating that the endoderm defects most likely occur prior to the 3- to 4-somite stage. These observations suggest that the endoderm differentiation defects in *Sox17* mutants may be responsible for the loss of LR asymmetry since the nodal flow and the subsequent signal transfer to the LPM occur between the 0- to 4-somite stages. In the next chapter, we examined the three steps of LR asymmetry in *Sox17* mutants to determine the specific step(s) when endoderm may be required for establishment of LR determination in normal embryos.

The left-right defects in *Sox17* mutants

Our analysis of *Sox17* mutant embryos at E9.5 revealed that the homozygous null mutants exhibited randomized heart looping orientation (Figure 2.6). This observation strongly suggested that loss of *Sox17* results in defects in LR determination. This was a novel discovery, since the initial description of *Sox17* mutants did not report this observation (Kanai-Azuma et al., 2002). In addition, a few mutant embryos also showed cardia bifida caused by the delayed convergence of foregut endoderm at the midline. The mutant embryos with fused heart fields formed a single heart tube that was shorter in length compared to age-matched control embryos (Figure 2.6A, B). Taken together these results suggested that defective endoderm differentiation in *Sox17* mutants not only affects heart morphology but also looping orientation (LR asymmetry).

We then investigated the expression pattern of heart-specific markers in *Sox17* mutants at E9.5. *Nkx2.5* is a homeobox gene that is expressed along the entire heart tube and forms the characteristic C-shaped looped orientation at 12- to 13-somite stages in control embryos (Lyons et al., 1995). In *Sox17* mutants, *Nkx2.5* was expressed in the heart tube that showed reduced rightward looping, however, the expression level was

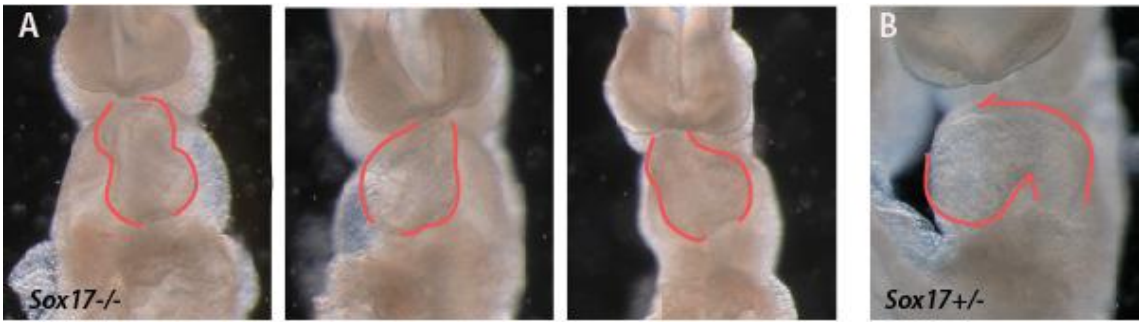


Figure 2.6 Randomized heart looping orientation in *Sox17* mutants. (A) Linear, left and right looped heart tubes in *Sox17* mutant embryos at E9.5 (shown in red lines). (B) Normal looping orientation (rightward) in *Sox17* heterozygous embryos.

lower than the control embryos, suggesting that endoderm defects due to loss of *Sox17* may affect cardiac differentiation (Figure 2.7A, A'). We also investigated *Nkx2.5* expression at the headfold stage to determine whether lack of *Sox17* affects differentiation of the heart precursors at the cardiac crescent. Again, *Nkx2.5* was expressed at a lower level than control embryos in the cardiac mesoderm, suggesting that SOX17 plays a role in heart induction (data not shown). These observations have been supported by other studies in ES cells that show the essential role of SOX17 in cardiac mesoderm specification (Liu et al., 2007).

Myosin light chain-2v (*Mlc2v*), a myosin gene specific for the ventricles is strongly expressed in the right and left ventricles in both control and *Sox17* mutant heart tubes, clearly revealing the unlooped straight heart shape in the mutant embryos (Figure 2.7B, B'; Tanaka et al., 1999). Our observations of the mutant heart phenotype suggests that in addition to the orientation of heart looping defect, the length of the heart tube was shorter compared to control embryos. Moreover, the anterior part of the heart tube, consisting of the outflow tract appeared to be particularly affected in the *Sox17* mutants; thus, we examined expression of outflow tract markers, *Cripto* and *Wnt11* (Cohen et al., 2012; Minchiotti et al., 2000). The expression pattern of both these genes showed the reduced size of the outflow tract in the mutant embryos (Figure 2.7C-D').

SOX17 protein is expressed in both embryonic and extraembryonically derived endoderm near the node

Prior to the onset of gastrulation, the blastocyst consists of epiblast cells covered by visceral endoderm cells, which play an important role in nutrient uptake and epiblast patterning (Bielinska et al., 1999; Rossant and Tam, 2009). It was previously thought that

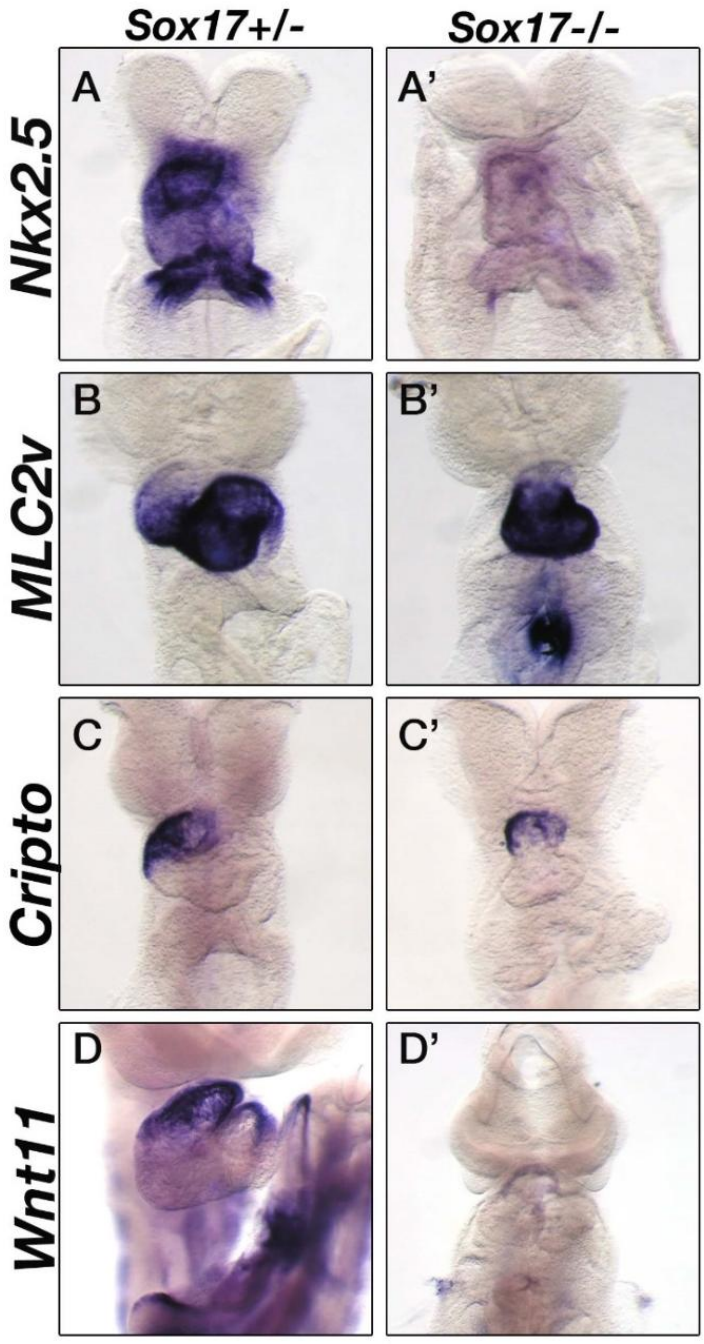


Figure 2.7 Defects in heart morphology and heart-specific marker expression in *Sox17* mutants. (A, A') *Nkx2.5* is expressed in the entire heart tube in the control embryo, but reduced in the *Sox17* mutants at 12- to 13-somite stage. (B, B') Ventricular marker *MLC2v* reveals the position of the ventricles in the looped heart in the control embryo and the straight heart in the *Sox17* mutants. (C, C') The outflow tract is marked by *Cripto* in the normal and *Sox17* mutant, revealing the smaller size in the linear heart tube in the mutant embryo. (D, D') *Wnt11* is another outflow tract marker that shows highly reduced expression in the unlooped heart in the *Sox17* mutant embryo.

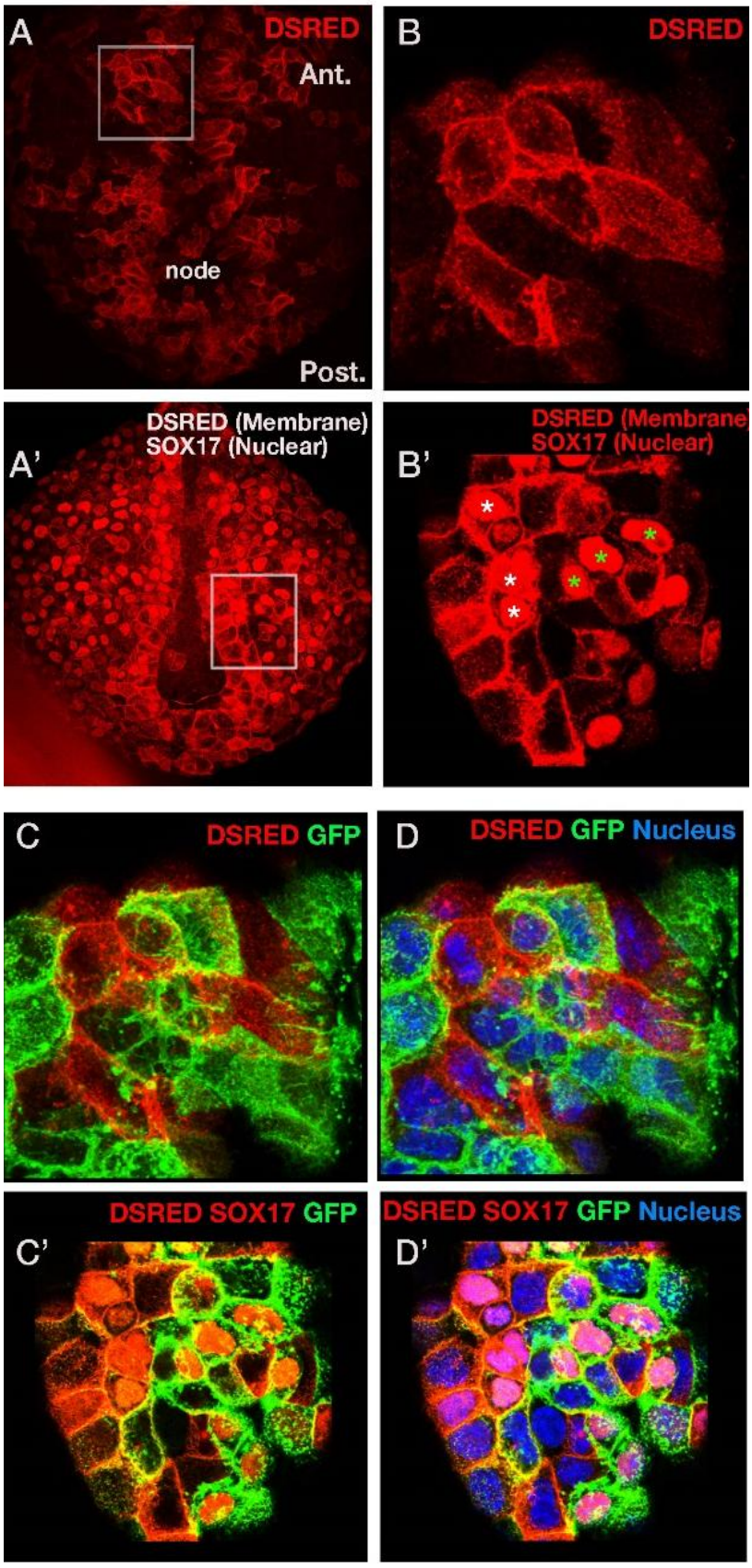
during gastrulation, the definitive endoderm arising from the epiblast displaces the entire visceral endoderm layer to the extraembryonic region to form a continuous epithelium. However, a recent study has shown that the definitive endoderm cells are integrated into the visceral endoderm layer and the visceral endoderm cells remain in the embryonic region and are assimilated into the gut tube (Kwon et al., 2008). In this report, Hadjantonakis and colleagues used an AFP-GFP transgenic line to label cells that were visceral endoderm in origin and traced their dispersal from the embryonic to the extraembryonic region as definitive endoderm emerged from the primitive streak. It was found that isolated AFP-GFP cells were interspersed amongst GFP-negative endoderm; however, a relatively higher proportion of AFP-GFP cells accumulated immediately adjacent to the node and the notochord at the early somite stages. It is important to note that the AFP-GFP cells in the embryonic region appeared similar in epithelial morphology to the definitive endoderm and did not express proteins characteristic of visceral endoderm, such as HNF4α (Kwon et al., 2008).

Our hypothesis involves investigation of the role of the gut endoderm in the determination of LR asymmetry; the presence of the visceral endoderm derived cells adjacent to the node suggested a potential role of these cells in the generation of LR asymmetry. We employed the *Sox2-cre* transgenic line, that express *Sox2-cre* specifically in epiblast cells, to distinguish between definitive endoderm cells derived from epiblast and the visceral endoderm by crossing with *Rosa dTdG (dTdG)* mice in which Cre recombination leads to a switch in the reporter gene expression from *DsRed* to *GFP* in recombined cells (Hayashi et al., 2002; Muzumdar et al., 2007). Hence, epiblast derived definitive endoderm expressed membrane GFP, whereas visceral endoderm derived cells

expressed DSRED in the membrane. Similar to the AFP-GFP localization, we found several DSRED expressing endoderm cells near the node and the notochord at the early somite stages (Figure 2.8A-D). This observation suggested that the endoderm layer lying between the node and the LPM was composed of a mixed population of both definitive endoderm and cells of visceral endoderm lineage.

The expression pattern of *Sox17* examined by *in situ* hybridization clearly reveals that *Sox17* is expressed in the endoderm adjacent to the node at the early somite stages (Figure 2.1). However, it was not clear whether *Sox17* was either expressed specifically in definitive endoderm or also in visceral endoderm derived cells. We then investigated the SOX17 protein localization by whole mount immunohistochemistry along with DSRED and GFP in *Sox2-cre/+;dTdG/+* embryos at the early somite stages. The available working primary antibodies for DSRED and SOX17 were both made in rabbit, preventing the use of separate secondary antibodies to detect DSRED and SOX17; however, the membrane and nuclear localization of DSRED and SOX17, respectively, allowed the two proteins to be distinguishable from each other. We found that some endoderm cells expressing DSRED in the membrane also expressed SOX17, although several DSRED positive cells were absent for SOX17 protein (Figure 2.8A'-B'). Similarly, a subset of GFP-positive cells expressed SOX17 protein, suggesting *Sox17* is not expressed in all definitive endoderm cells near the node (Figure 2.8C'-D'). The SOX17 protein localization in the visceral endoderm derived DSRED positive cells clearly indicates that *Sox17* expression is activated in these cells due to induction from neighboring definitive endoderm. The data also strongly suggest that both definitive and visceral endoderm cells are similar in epithelial characteristics and are likely to behave

Figure 2.8 SOX17 protein localization in embryonic and extraembryonic endoderm at early somite stages. (A, B) DSRED, a red fluorescent protein, (shown in red) localization is seen in extraembryonic endoderm cells in *Sox2cre/+;dT/+* embryos. B represents magnified image of the highlighted part of the embryo in A. (C, D) Green fluorescent protein (GFP) (shown in green) is localized in definitive endoderm derived from the epiblast. Nuclei are shown in blue. (A', B') Both SOX17 (nuclear, red) and DSRED (membrane, red) were visualized in the same embryo. SOX17 nuclear localization in extraembryonic endoderm is shown with white stars, whereas green stars represent SOX17 localization in the endoderm of epiblast origin (B'). (C', D') The embryonic endoderm and nucleus (blue) are shown with GFP localization (green).


A
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C'
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uniformly in the entire region between the node and the LPM. Thus, *Sox17* acts as an important regulator of all endoderm cells adjacent to the node.

We then examined whether the role of *Sox17* in endoderm differentiation encompassed both the definitive endoderm and the endoderm cells of extraembryonic lineage found to be present in the embryonic region (Figure 2.4; Kwon et al., 2008). We generated conditional mutants containing the *Sox2-cre* transgene and homozygous floxed *Sox17* allele to test this possibility. In these conditional mutants, *Sox17* was only deleted in definitive endoderm cells due to *Sox2-cre* activity specifically in the epiblast which contains the primitive streak, but remained wildtype in the endoderm cells of extraembryonic origin. We found that *Foxa2*, expressed strongly in the foregut and the hindgut pockets, was similarly reduced in both *Sox17* null and *Sox2-cre* conditional mutants (Figure 2.9). This result suggested that loss of *Sox17* function only in the epiblast derived definitive endoderm cells either affected the entire endoderm morphology uniformly or contribution of wildtype endoderm cells of extraembryonic origin was relatively minor. It is likely that *Sox17* expression in the definitive endoderm from the poststreak stages also influences differentiation of the neighboring extraembryonic cells in the embryonic foregut and hindgut regions. Moreover, SOX17 protein was found to be induced in the cells in the extraembryonic endoderm cells near the node (Figure 2.8A'-B'). The randomized heart looping and embryonic turning in conditional *Sox17* mutants is similar to the null mutants, suggesting a similar degree of LR defects in the conditional mutants relative to null mutants. *Shh* expression in *Sox17* conditional mutants and null mutants also exhibited comparable foregut and hindgut defects as well as LR abnormalities (data not shown).

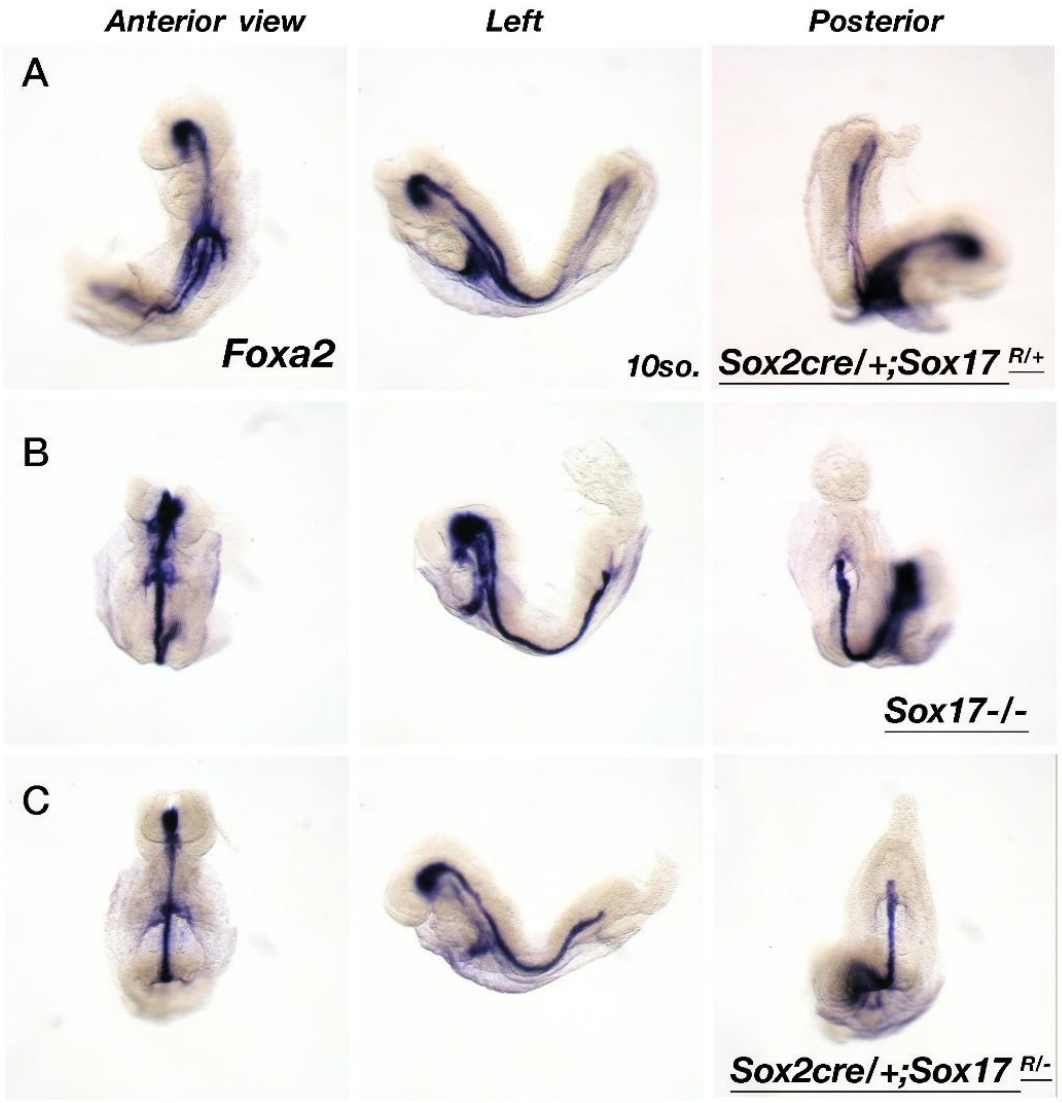

Figure 2.9 *Foxa2* expression in the foregut and hindgut in *Sox17* conditional (epiblast-deleted) mutants is similar to *Sox17* null mutants. In *Sox2cre/+;Sox17*$^{R/+}$ control embryos (A), *Foxa2* is expressed broadly in the foregut and the hindgut, whereas in the *Sox17* null mutant (B) and *Sox2cre/+;Sox17*$^{R/-}$ conditional mutant (C), *Foxa2* expression is reduced in the foregut and highly attenuated in the hindgut at the 10-somite stage.

Summary

Here, we have investigated the expression profile of the endoderm regulator, *Sox17,* during the early gastrulation stages and observed the endoderm differentiation defects associated with loss of *Sox17* function. *Sox17* was expressed in the endoderm emerging from the primitive streak as well as in the endoderm in close proximity to the node, which is involved in the breaking of LR symmetry. In contrast, the other endoderm expressed genes; *Foxa2, Cer-1* and *Hex* did not show expression in the endoderm cells adjoining the node. We found that endoderm markers such as *Foxa2* and *Hex* showed a reduced extent of expression in the foregut and the hindgut region in *Sox17* mutants. The endoderm defect was most severe at the level of the node position in the mid-embryonic region at 4- to 7-somite stages, indicated by the complete loss of *Nepn* expression in the mutant embryos. The other cell types, such as mesoderm and ectoderm, appeared relatively normal at the early somite stages; however, the randomized heart looping orientation suggested that loss of *Sox17* resulted in defects in LR determination. SOX17 protein was found to be localized not only in the epiblast derived definitive endoderm but also in endoderm cells derived from the extraembryonic origin at the early somite stages, suggesting that SOX17 may be required for differentiation of both types of endoderm cells. The observed similarities in *Foxa2* and *Shh* expression pattern defects in both the *Sox2-cre* deleted *Sox17* conditional and the *Sox17* null mutants supported this possibility. Thus, these studies have clearly established a fundamental role for *Sox17* in the regulation of endoderm differentiation during the stages when LR determination takes place. In the next chapter, we explore the structural and functional aspects of the different steps in LR determination, as mentioned in Chapter 1, in the *Sox17* mutants to uncover

the precise requirement of gut endoderm in the establishment of LR asymmetry.

Materials and Methods

Mice and embryo collection

Mice carrying the *Sox17* null allele (Kanai-Azuma et al., 2002) and the *Sox17*^{GFP} allele (Kim et al., 2007) were maintained in a mixed genetic background of 129sv, C57BL/6J and CD-1, and 129sv and C57BL/6J, respectively. Prior to harvest, the stages of embryos were monitored and estimated by ultrasound scan of the pregnant mother (Visual Sonics). Embryos were genotyped as described previously (Kanai-Azuma et al., 2002; Kim et al., 2007). All procedures using mice were in accordance of the IACUC protocol at The University of Utah.

Whole-mount *in situ* hybridization, immunohistochemistry and image processing

Whole-mount *in situ* hybridization was performed according to standard procedures using the following probes: *Sox17*, a combination of 0.8 kb and 0.6 kb fragments that cover the *Sox17* coding region. Following whole-mount *in situ* hybridization, embryos were imaged in 80% glycerol and 10 μm sections were prepared by standard paraffin sectioning. For immunohistochemistry (IHC), embryos at E7.5-8.5 were fixed in 4% paraformaldehyde (PFA) in PBS for 1 hour at room temperature (RT), dehydrated through a methanol series and stored at –20°C until use. Primary antibodies used for IHC: GFP (Invitrogen A21311, 1:1000), DSRED (Clontech 632543, 1:50) and SOX17 (Gift from Kanai-Azuma, 1:100) Alexa Fluor dye-labeled secondary antibodies were used (Invitrogen, 1:1000). Nuclei were counterstained with DAPI.

References

Ang, S. L. and Rossant, J. (1994). HNF-3 beta is essential for node and notochord formation in mouse development. *Cell* **78**(4), 561-74.

Belo, J. A., Bachiller, D., Agius, E., Kemp, C., Borges, A. C., Marques, S., Piccolo, S. and De Robertis, E. M. (2000). Cerberus-like is a secreted BMP and nodal antagonist not essential for mouse development. *Genesis* **26**(4), 265-70.

Biben, C., Stanley, E., Fabri, L., Kotecha, S., Rhinn, M., Drinkwater, C., Lah, M., Wang, C. C., Nash, A., Hilton, D. et al. (1998). Murine cerberus homologue mCer-1: a candidate anterior patterning molecule. *Dev Biol* **194**(2), 135-51.

Bielinska, M., Narita, N. and Wilson, D. B. (1999). Distinct roles for visceral endoderm during embryonic mouse development. *Int J Dev Biol* **43**(3), 183-205.

Burtscher, I. and Lickert, H. (2009). Foxa2 regulates polarity and epithelialization in the endoderm germ layer of the mouse embryo. *Development* **136**(6), 1029-38.

Cohen, E. D., Miller, M. F., Wang, Z., Moon, R. T. and Morrisey, E. E. (2012). Wnt5a and Wnt11 are essential for second heart field progenitor development. *Development* **139**(11), 1931-40.

Feistel, K. and Blum, M. (2008). Gap junctions relay FGF8-mediated right-sided repression of Nodal in rabbit. *Dev Dyn* **237**(12), 3516-27.

Franklin, V., Khoo, P. L., Bildsoe, H., Wong, N., Lewis, S. and Tam, P. P. (2008). Regionalisation of the endoderm progenitors and morphogenesis of the gut portals of the mouse embryo. *Mech Dev* **125**(7), 587-600.

Hayashi, S., Lewis, P., Pevny, L. and McMahon, A. P. (2002). Efficient gene modulation in mouse epiblast using a Sox2Cre transgenic mouse strain. *Gene Expr Patterns* **2**(1-2), 93-7.

Hou, J., Charters, A. M., Lee, S. C., Zhao, Y., Wu, M. K., Jones, S. J., Marra, M. A. and Hoodless, P. A. (2007). A systematic screen for genes expressed in definitive endoderm by Serial Analysis of Gene Expression (SAGE). *BMC Dev Biol* **7**, 92.

Kaestner, K. H., Hiemisch, H., Luckow, B. and Schutz, G. (1994). The HNF-3 gene family of transcription factors in mice: gene structure, cDNA sequence, and mRNA distribution. *Genomics* **20**(3), 377-85.

Kanai-Azuma, M., Kanai, Y., Gad, J. M., Tajima, Y., Taya, C., Kurohmaru, M., Sanai, Y., Yonekawa, H., Yazaki, K., Tam, P. P. et al. (2002). Depletion of definitive gut endoderm in Sox17-null mutant mice. *Development* **129**(10), 2367-79.

Kim, I., Saunders, T. L. and Morrison, S. J. (2007). Sox17 dependence distinguishes the transcriptional regulation of fetal from adult hematopoietic stem cells. *Cell* **130**(3), 470-83.

Kwon, G. S., Viotti, M. and Hadjantonakis, A. K. (2008). The endoderm of the mouse embryo arises by dynamic widespread intercalation of embryonic and extraembryonic lineages. *Dev Cell* **15**(4), 509-20.

Lee, C. S., Friedman, J. R., Fulmer, J. T. and Kaestner, K. H. (2005). The initiation of liver development is dependent on Foxa transcription factors. *Nature* **435**(7044), 944-7.

Levin, M. and Mercola, M. (1999). Gap junction-mediated transfer of left-right patterning signals in the early chick blastoderm is upstream of Shh asymmetry in the node. *Development* **126**(21), 4703-14.

Liu, J. K., DiPersio, C. M. and Zaret, K. S. (1991). Extracellular signals that regulate liver transcription factors during hepatic differentiation in vitro. *Mol Cell Biol* **11**(2), 773-84.

Liu, Y., Asakura, M., Inoue, H., Nakamura, T., Sano, M., Niu, Z., Chen, M., Schwartz, R. J. and Schneider, M. D. (2007). Sox17 is essential for the specification of cardiac mesoderm in embryonic stem cells. *Proc Natl Acad Sci USA* **104**(10), 3859-64.

Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L. and Harvey, R. P. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. *Genes Dev* **9**(13), 1654-66.

Martinez Barbera, J. P., Clements, M., Thomas, P., Rodriguez, T., Meloy, D., Kioussis, D. and Beddington, R. S. (2000). The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation. *Development* **127**(11), 2433-45.

McGrath, J., Somlo, S., Makova, S., Tian, X. and Brueckner, M. (2003). Two populations of node monocilia initiate left-right asymmetry in the mouse. *Cell* **114**(1), 61-73.

Minchiotti, G., Parisi, S., Liguori, G., Signore, M., Lania, G., Adamson, E. D., Lago, C. T. and Persico, M. G. (2000). Membrane-anchorage of Cripto protein by glycosylphosphatidylinositol and its distribution during early mouse development. *Mech Dev* **90**(2), 133-42.

Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L. and Luo, L. (2007). A global double-fluorescent Cre reporter mouse. *Genesis* **45**(9), 593-605.

Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T. and

De Robertis, E. M. (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* **397**(6721), 707-10.

Rossant, J. and Tam, P. P. (2009). Blastocyst lineage formation, early embryonic asymmetries and axis patterning in the mouse. *Development* **136**(5), 701-13.

Tam, P. P., Kanai-Azuma, M. and Kanai, Y. (2003). Early endoderm development in vertebrates: lineage differentiation and morphogenetic function. *Curr Opin Genet Dev* **13**(4), 393-400.

Tam, P. P., Khoo, P. L., Lewis, S. L., Bildsoe, H., Wong, N., Tsang, T. E., Gad, J. M. and Robb, L. (2007). Sequential allocation and global pattern of movement of the definitive endoderm in the mouse embryo during gastrulation. *Development* **134**(2), 251-60.

Tanaka, M., Chen, Z., Bartunkova, S., Yamasaki, N. and Izumo, S. (1999). The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. *Development* **126**(6), 1269-80.

Tanaka, Y., Okada, Y. and Hirokawa, N. (2005). FGF-induced vesicular release of Sonic Hedgehog and Retinoic acid in leftward nodal flow is critical for left-right determination. *Nature* **435**(7039), 172-7.

Thomas, P. Q., Brown, A. and Beddington, R. S. (1998). Hex: a homeobox gene revealing peri-implantation asymmetry in the mouse embryo and an early transient marker of endothelial cell precursors. *Development* **125**(1), 85-94.

Wilson, V. and Beddington, R. S. (1996). Cell fate and morphogenetic movement in the late mouse primitive streak. *Mech Dev* **55**(1), 79-89.

CHAPTER 3

GUT ENDODERM IS INVOLVED IN TRANSFER OF LEFT RIGHT ASYMMETRY FROM THE NODE TO THE LATERAL PLATE MESODERM IN THE MOUSE EMBRYO

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Keywords: Left right asymmetry; endoderm; gap junction; Sox17; Nodal

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Summary

In the mouse, the initial signals that establish left-right (LR) asymmetry are determined in the node by nodal flow. These signals are then transferred to the lateral plate mesoderm through cellular and molecular mechanisms that are not well characterized. We hypothesized that endoderm may play a role in this process because: (1) endoderm is tightly apposed to the node and covers the outer surface of the embryo and; (2) just after nodal flow is established, higher Ca^{2+} flux has been reported on the left side near the node, most likely in the endoderm cells. Here, we studied the role of endoderm cells in this process by analyzing mouse *Sox17* null mutant embryos which possess endoderm-specific defects. *Sox17*$^{-/-}$ embryos showed no expression or significantly reduced expression of LR asymmetric genes in the left lateral plate mesoderm. In *Sox17* mutant endoderm, the localization of connexin proteins on the cell membrane was greatly reduced, resulting in defective gap junction formation, which seemed to be caused by incomplete development of organized epithelial structures. Our findings suggest an essential role of endoderm cells in the signal transfer step from the node to the LPM, possibly using gap junctional communication to establish the LR axis of the mouse.

Introduction

Establishing a three-dimensional axis, with anteroposterior, dorsoventral and left-right (LR) coordinates, is a critical step in embryogenesis. LR axis determination provides positional information for internal organs after the other two axes are established (Beddington and Robertson, 1999). The process of establishing LR asymmetry in the mouse occurs in four steps (Hamada et al., 2002). First, symmetry is

broken in the node by a leftward flow of extraembryonic fluid termed nodal flow (Nonaka et al., 1998). Second, the LR signal established in the node is transferred from the node to the lateral plate mesoderm (LPM), which comprises the mesodermal component of internal organs. Third, *Nodal* begins to be expressed in the LPM near the node and then extends rostrocaudally, expanding throughout the entire left LPM. This expansion is achieved by positive and negative transcriptional feedback loops between *Nodal* and *Lefty1/2*, together with the long-range displacement of the secreted proteins NODAL, LEFTY1 and LEFTY2 (Marjoram and Wright, 2011; Osada et al., 2000; Saijoh et al., 2000). NODAL also turns on the *Pitx2* transcription factor in the left LPM. Finally, the NODAL signal and *Pitx2* establish characteristics of the left side in the LPM, and asymmetric morphogenesis takes place in internal organs.

Although the mechanisms of LR determination have been extensively analyzed, it is still unclear how LR signals in the node are transferred to the left LPM. Bilateral expression of *Nodal* in the peripheral region of the node (in crown cells or perinode) is considered to be essential for this step, as its depletion in this region results in the loss of *Nodal* expression in the LPM (Brennan et al., 2002; Saijoh et al., 2003). Considering the fact that NODAL is a secreted molecule that can diffuse over long distances, NODAL protein generated in the node might travel to the LPM through the extracellular matrix (ECM), thereby transferring LR information to this region. In support of this contention, Oki et al. (2007) demonstrated that sulfated ECM proteoglycans such as chondroitin sulfate and heparan sulfate are necessary to induce *Nodal* expression in the LPM. However, there is no direct evidence to support the movement of NODAL from the node to the LPM. Collectively, these data suggest that expression of NODAL protein in the

crown cells is essential for *Nodal* expression in the left LPM; however, it is unclear whether NODAL is itself the signal. Consequently, both the molecular nature of the LR signal and the mechanism by which this signal is transferred to the LPM remain to be determined. Several studies have reported that after nodal flow is established, higher Ca^{2+} flux is observed on the left side near the node, most likely in the endoderm cells (Hadjantonakis et al., 2008; McGrath et al., 2003; Tanaka et al., 2005). This observation and the fact that endoderm in the mouse embryo is located adjacent to the node and covers the entire LPM at that stage, led us to hypothesize that endoderm might play a role in the signal transfer from the node to the left LPM.

To address the roles of endoderm in LR determination, we analyzed *Sox17* mutant mice. SOX17 is a high-mobility group transcription factor and is specifically expressed in the endoderm and endothelial cells of developing embryos (Kanai-Azuma et al., 2002; Sakamoto et al., 2007). Null mutants die at around embryonic day (E) 10, with an abnormal heart and loss of blood stem cells; however, at E8.0, when LR determination occurs, the*Sox17* mutants have defects specifically in endoderm cells (Kanai-Azuma et al., 2002; Kim et al., 2007; Pfister et al., 2011; Sakamoto et al., 2007).

In this study, we found that a majority of *Sox17*$^{-/-}$ embryos fail to express *Nodal*, *Lefty1/2* and *Pitx2* in the LPM, despite the fact that LR determination within the node of *Sox17* mutant embryos was essentially normal. We also demonstrated that the LPM has the potential to express these asymmetric genes in *Sox17*$^{-/-}$ embryos. Our data suggest that the primary LR defect in *Sox17*$^{-/-}$ embryos is an impaired ability to transfer the LR signal from the node to the LPM. Significantly, membrane localization of gap junction connexin proteins was impaired in *Sox17*$^{-/-}$ endoderm, resulting in defective

intercellular transport between endoderm cells. Collectively, our findings are the first to suggest an essential role of endoderm cells in the signal transfer step from the node to the LPM, possibly using gap junction communication to establish the LR axis of the mouse.

Materials and methods

Mice and embryo collection

Mice carrying the *Sox17* null allele (Kanai-Azuma et al., 2002) and the *Sox17*^{GFP} allele (Kim et al., 2007) were maintained in a mixed genetic background of 129sv, C57BL/6J and CD-1, and 129sv and C57BL/6J, respectively. Prior to harvest, the stages of embryos were monitored and estimated by ultrasound scan of the pregnant mother (Visual Sonics). Embryos were genotyped as described previously (Kanai-Azuma et al., 2002; Kim et al., 2007). All procedures using mice were in accordance of the IACUC protocol at The University of Utah.

Whole-mount in situ hybridization, immunohistochemistry and image processing

Whole-mount in situ hybridization was performed according to standard procedures (Wilkinson, 1998) using the following probes: *Sox17*, a combination of 0.8 kb and 0.6 kb fragments that cover the *Sox17* coding region, and previously described probes (Kanai et al., 1996); *Nodal*, a combination of three plasmids containing 5′ and 3′ untranslated regions (UTR) and the coding sequence; mouse *Cx43* mRNA was detected using a rat 1125 bp cDNA fragment that shares 95% sequence identity with mouse *Cx43* (Beahm et al., 2006); *Cerl2* (Hashimoto et al., 2004); *Cryptic* (Shen et al., 1997); *Foxf1* (Mahlapuu et al., 2001); *Foxh1*(Saijoh et al., 2000); *Gdf1* (Rankin et al.,

2000); *Lefty1/Lefty2* (Meno et al., 1996), which cross-hybridizes with both *Lefty1* and *Lefty2* mRNA; *L-Plunc* (Hou et al., 2004); *Meox1* (Candia et al., 1992); *Pitx2* (Yoshioka et al., 1998) and *Raldh2* (Niederreither et al., 1997). Following whole-mount in situ hybridization, embryos were imaged in 80% glycerol and 10 μm sections were prepared by standard paraffin sectioning.

For immunohistochemistry (IHC), embryos at E7.5-8.5 were fixed in 4% paraformaldehyde (PFA) in PBS for 1 hour at room temperature (RT), dehydrated through a methanol series and stored at –20°C until use. Primary antibodies used for IHC: acetylated tubulin (Sigma clone 6-11B-1, 1:200); β-galactosidase (Abcam ab9361, 1:500); chondroitin sulfate (Sigma CS-56, 1:100); CX40 (Santa Cruz sc-20466, 1:100); CX43 (Sigma C6219, 1:500); E-cadherin (BD Transduction 610181, 1:100); γ-tubulin (Sigma T3559, 1:100); GFP (Invitrogen A21311, 1:1000); laminin (Sigma L9393, 1:100) and ZO1 (Zymed 33-9100, 1:400). Alexa Fluor dye-labeled secondary antibodies were used (Invitrogen, 1:1000). Nuclei were counterstained with DAPI.

Confocal microscopy images were obtained at 0.3-1 μm intervals using a Leica TCS SP5 system. Images were processed using FluoRender software (http://www.sci.utah.edu/software/13-software/127-fluorender.html). Fluorescent signals are pseudo-colored in the figures to permit viewing by the color-blind.

Scanning electron microscopy

Embryos dissected at E8.0 were fixed in 2.5% glutaraldehyde/1% PFA in 0.1 M Sorensen's phosphate buffer (pH 7.4) at RT overnight, washed in 0.1 M cacodylate buffer (pH 7.4), and postfixed in 1% osmium tetroxide in cacodylate buffer for 45 minutes at RT. Embryos were then dehydrated through a graded ethanol series, critical point dried,

gold coated, and mounted on a low vacuum electron microscope (Hitachi S-2460N).

Nodal flow

To assess nodal flow over the node surface, FluorSpheres (0.5 μm, Invitrogen) were added to the medium and the embryo was mounted on a slide-glass chamber under a time-lapse fluorescence microscope kept in a chamber at 37°C. The directional movements and velocities of the beads across the node surface were recorded at 0.2-second intervals and traced using the MTrackJ plug-in in ImageJ (NIH).

Iontophoresis of whole embryos

Embryos were mounted in a fixed position on a silicon-derived embryo-holding chamber containing HEPES-buffered DMEM. Embryos were visualized at 40× using an Olympus BX-WI fixed stage microscope. A glass micropipette containing a filtered solution of 0.1 M LiCl, 5% Lucifer Yellow and 1% Rhodamine dextran (10 kDa) was positioned within an endodermal cell using a micromanipulator. The iontophoresis was performed using an Axopatch 200A patch clamp and Digidata 1320A (Axon Instruments) at an iontophoretic current of –1.0 nA and a negative holding voltage of 60 mV generating a 25-30 MΩ circuit resistance. After a 10-second iontophoresis of dyes, the micropipette was removed and epifluorescence and bright-field images were captured using a MicroFire M digital camera (Olympus).

Nodal expression is absent in the LPM of *Sox17* mutant embryos

We have previously shown that *Sox17*$^{-/-}$ embryos do not undergo embryonic turning and most mutants have aberrant heart looping (Kanai-Azuma et al., 2002; Pfister

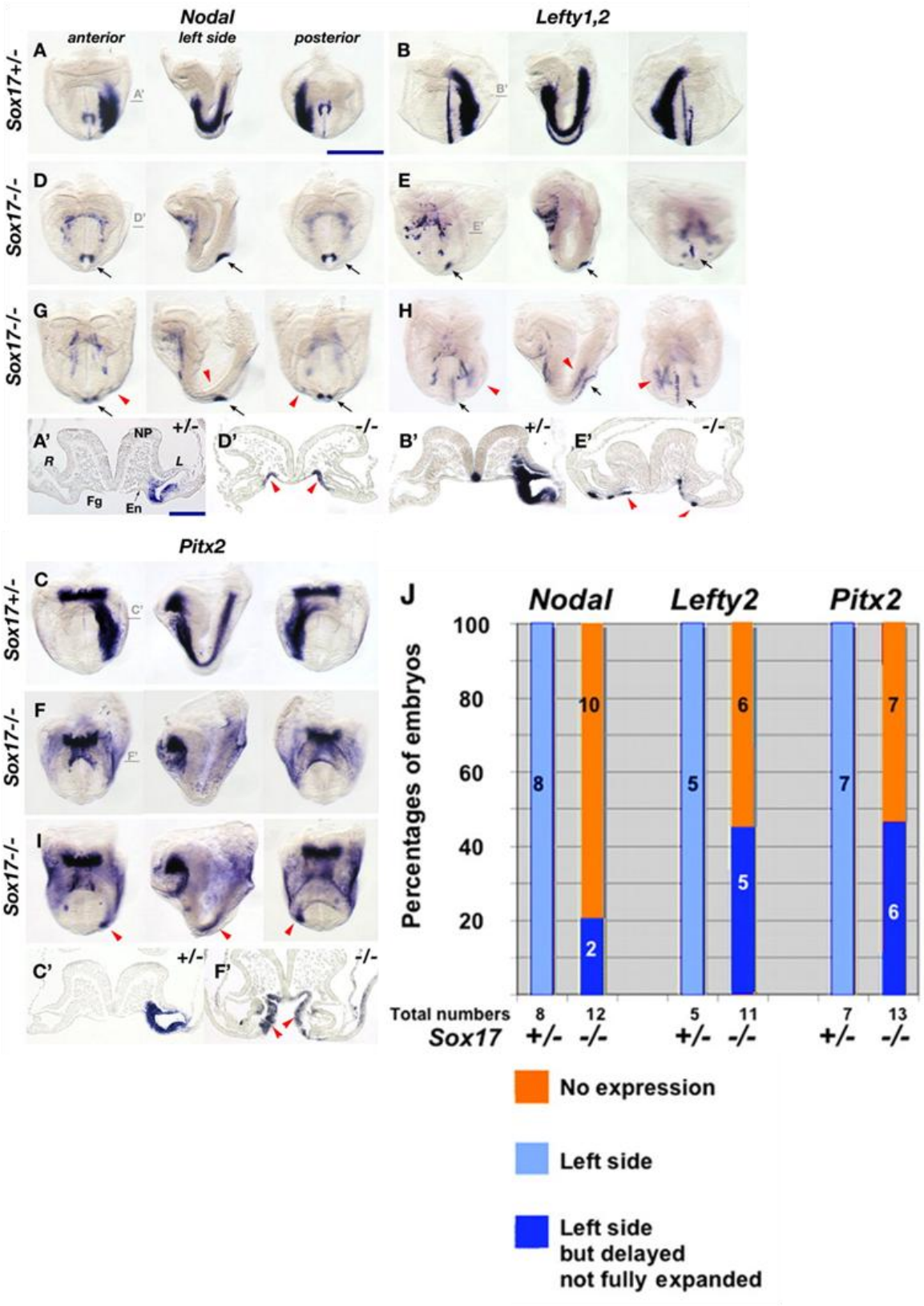
et al., 2011; Sakamoto et al., 2007). Further observations (data not shown) suggested that heart looping in *Sox17*$^{-/-}$ embryos is essentially random. Since both embryonic turning and heart looping are readouts of LR patterning, these data suggested that *Sox17*$^{-/-}$ embryos have a defect in LR determination. We therefore examined the expression of several genes that are required for LR establishment in *Sox17*$^{-/-}$ embryos.

We first examined *Nodal*, which is a master gene for LR determination (Hamada et al., 2002). In *Sox17*$^{-/-}$ embryos, *Nodal* expression was absent from the entire LPM (10/12) or was weakly expressed in a small region of the left LPM near the node (2/12). In *Sox17*$^{+/-}$ (*n*=8) and wildtype (*n*=22) embryos, *Nodal* was expressed throughout the left LPM (Figure 3.1A, D, G, J). Despite its absence in the LPM, perinodal expression of *Nodal* was essentially normal in *Sox17*$^{-/-}$ embryos (Figure 3.1D, G, black arrows).

We next examined two other left-specific genes, *Lefty2* and *Pitx2*, that are downstream targets of NODAL (Osada et al., 2000; Saijoh et al., 2000). As expected, both *Lefty2* and *Pitx2* were either completely absent (6/11, 7/13, respectively) or were expressed in a restricted region of the left LPM near the node (5/11, 6/13, respectively) in *Sox17*$^{-/-}$embryos at a stage when they are expressed throughout the entire left LPM in both *Sox17*$^{+/-}$ and wildtype embryos (Figure 3.1B, C, E, F, H-J). We did not observe right side or bilateral expression of these genes in *Sox17*$^{-/-}$ embryos, suggesting defective but not randomized LR determination in *Sox17*$^{-/-}$ embryos. In addition, we unexpectedly observed that *Nodal*, *Lefty2* and *Pitx2* were ectopically expressed in the foregut endoderm of *Sox17*$^{-/-}$ embryos at the early somite stage (Figure 3.1A′-F′, red arrowheads).

This expression pattern of *Sox17* (Chapter 2, Figure 2.1) suggests three possible

Figure 3.1 Loss or reduction of the LR asymmetric gene expression in *Sox17* mutants. Expression patterns of *Nodal* (A, D, G), *Lefty1*/*Lefty2* (B, E, H) and *Pitx2* (C, F, I) in *Sox17*$^{+/-}$ (A-C) and *Sox17*$^{-/-}$ (D-I) embryos at E8.2. (D-E) the *Sox17*$^{-/-}$ embryos show loss of asymmetric gene expression in the LPM. (G-H) The asymmetric gene expression was limited to a small region near the node in the *Sox17*$^{-/-}$ embryos (red arrowheads in G, H, I). Ectopic expression domains of *Nodal*, *Lefty2*, and *Pitx2* were observed in the endoderm cells (red arrowheads in D', E', F'). *Lefty1* expression in the node is indicated by black arrows in E and H. *Nodal* expression in the node is shown by black arrows (D, G). A'-F' sections, the positions are indicated by lines in A-F. (J) Ratio of LR gene expression in the LPM. Fg, foregut; Hg, hindgut; NP, neural plate. Scale bars: 500 μm (A-I), 200 μm (A'-F').


Nodal
Lefty1,2
anterior
left side
posterior
Sox17+/-
Sox17-/-
Sox17-/-
A
B
D
E
G
H
A'
B'
D'
E'
NP
R
L
Fg
En
+/-
-/-
Pitx2
C
F
I
C'
F'
J
Nodal
Lefty2
Pitx2
Percentages of embryos
100
80
60
40
20
8
10
2
5
6
5
7
7
6
Total numbers
8
12
5
11
7
13
Sox17
+/-
-/-
+/-
-/-
+/-
-/-
No expression
Left side
Left side
but delayed
not fully expanded

explanations for the LR determination defects observed in *Sox17*$^{-/-}$ embryos: (1) the initial LR determination in the node is defective; (2) the transfer of LR signals from the node to the left LPM is impaired; or (3) the defective LPM cannot express or amplify *Nodal* after the LR signal from the node is received.

Sox17 mutants can respond to Nodal signaling in the LPM

First, we examined the possibility that *Sox17*$^{-/-}$ LPM has an impaired ability to respond to Nodal signaling. We examined LPM expressed genes including NODAL signal components in *Sox17*$^{-/-}$ embryos, including *Foxf1*, a key gene for LPM differentiation (Figure 3.2A, E; Tsiairis and McMahon, 2009), the coreceptor *Cryptic* (*Cfc1* – Mouse Genome Informatics) (Figure 3.2B, F; Yan et al., 1999), the transcription factor *Foxh1* (Figure 3.2C, G; Saijoh et al., 2000), and the coligand of NODAL, *Gdf1* (Figure 3.2D, H; Rankin et al., 2000; Tanaka et al., 2007). All of these genes were expressed in *Sox17*$^{-/-}$ embryos with expression patterns similar to those found in *Sox17*$^{+/-}$ control embryos (Figure 3.2A-H).

To directly test whether the LPM in *Sox17* mutants can express *Nodal* in response to Nodal signaling, exogenous introductions of *Nodal* expression were performed (Figure 3.2I-O). *Nodal* expression is regulated by a positive-feedback mechanism (Osada et al., 2000; Saijoh et al., 2000); thus, if the LPM in *Sox17*$^{-/-}$ embryos has an ability to respond to Nodal signaling, exogenous *Nodal* should be able to induce endogenous *Nodal* expression in the LPM of *Sox17*$^{-/-}$ embryos. *Nodal* expression vectors were introduced into a small area of the right LPM together with *GFP* expression vectors as a reporter of transfected cells, using a lipofection technique (Figure 3.2I-K; Nakamura et al., 2006). Both the control LPM (6/6; Figure 3.2L, M) and the *Sox17*$^{-/-}$ LPM (5/5; Figure 3.2N, O)

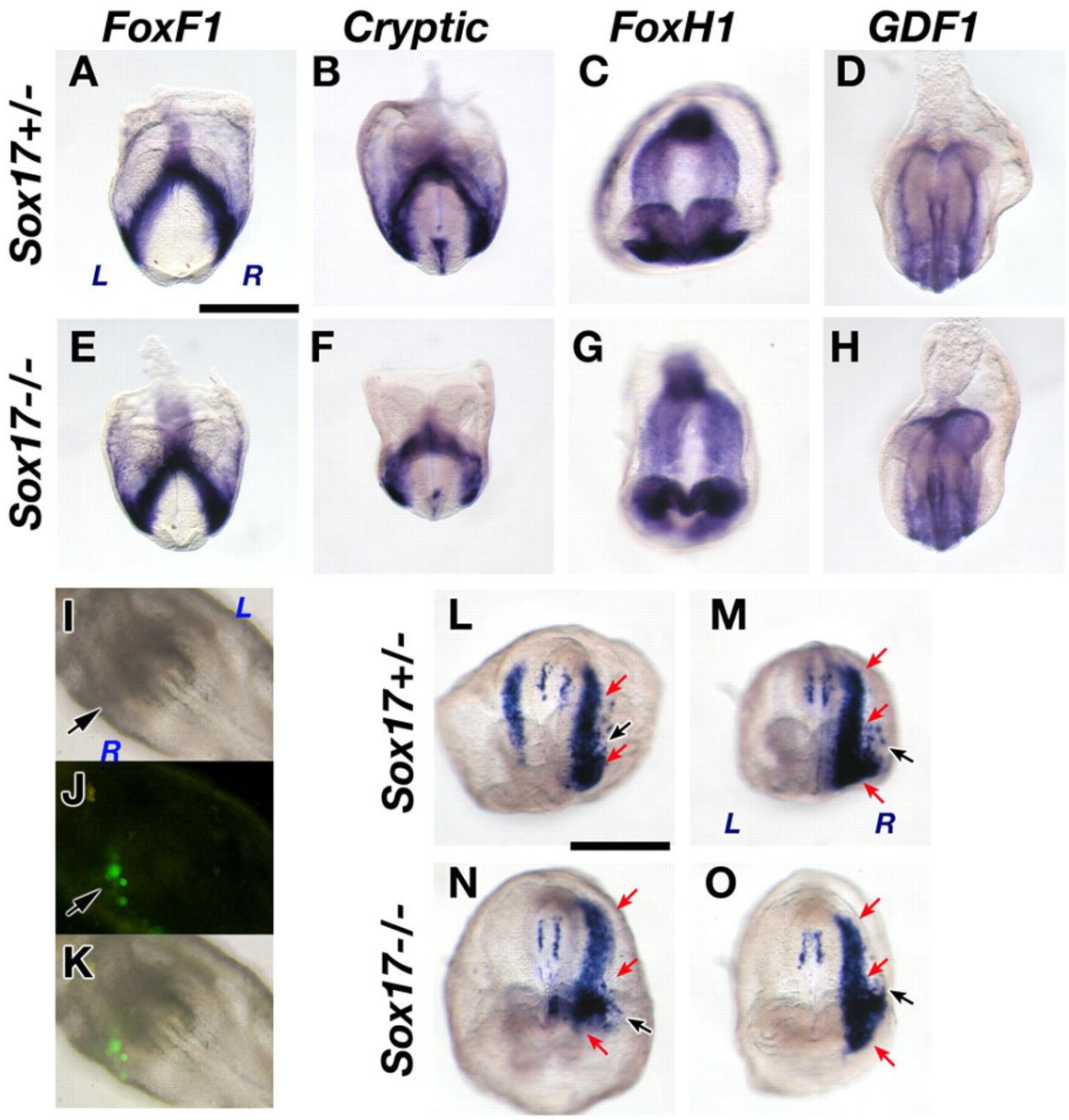


Figure 3.2 Lateral plate mesoderm is properly specified in the *Sox17* mutants. (A, E) *FoxF1*, (B, F) *Cryptic*, (C, G) *FoxH1*, (D, H) *Gdf1* were normally expressed in the $Sox17^{+/-}$ and $Sox17^{-/-}$ embryos at the 5- to 6-somite stage. (J-O) Induction of endogenous *Nodal* expression by ectopic Nodal activity in the right LPM. Lipofection mixture of the *Nodal* and *GFP* expression vectors was injected into the right LPM (arrow in I) at headfold stage. GFP-expressing cells represent cells that expressed exogeneous *Nodal* (arrow in J). Induced endogenous *Nodal* was expressed on the right side in the $Sox17^{+/-}$ (red arrows in L, M) and in $Sox17^{-/-}$ embryos (red arrows in N, O). Black arrows in L-O indicate the sites of exogenous *Nodal*. Note that the left side expression was reduced (L) or completely repressed (M) by the right side expression of *Nodal* in the $Sox17^{+/-}$ embryos. Scale bars: 500 μm.

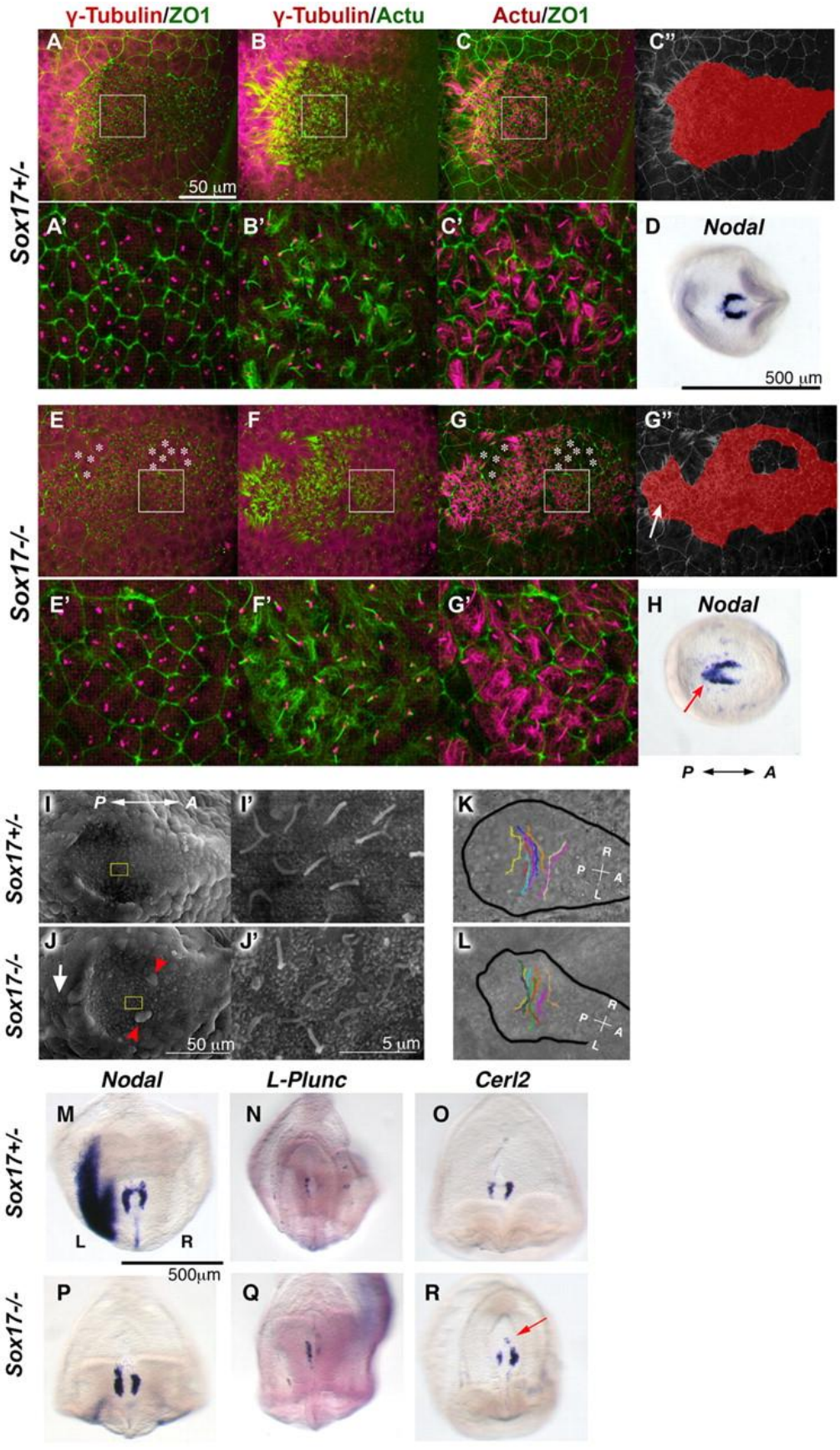
expressed *Nodal* throughout the right LPM in response to ectopic NODAL activity in this small region. These data suggested that the LPM in *Sox17*$^{-/-}$ embryos is capable of initiating and expanding *Nodal* expression when supplied with a source of NODAL.

Node formation is partially affected but LR determination at the node appears to be normal in *Sox17* mutants

Next, we examined the possibility that the initial LR determination in the node is affected in *Sox17*$^{-/-}$ embryos. The node precursors are formed at the most anterior part of the primitive streak, which is covered by endoderm cells (Chapter 2, Figure 2.1B, B′). Hence, these two tissues could interact substantially with each other during development, and endoderm may affect the differentiation of the node precursors (Sulik et al., 1994). In *Sox17*$^{-/-}$embryos, *Nodal* was expressed in the perinodal region (Figure 3.1D, G; black arrows), as observed in *Sox17*$^{+/-}$ embryos; however, expression at the headfold stage was extended more posteriorly in *Sox17*$^{-/-}$ embryos (Figure 3.3H, arrow), whereas expression in control embryos was horseshoe shaped (Figure 3.3D).

Acetylated tubulin (cilia) and γ-tubulin (basal bodies) immunohistochemistry (IHC) showed that cilia formed normally in the *Sox17*$^{-/-}$ node, although cilia formation was also observed in segregated node-like cells found posterior to the node (Figure 3.3A-G, arrow in 3.3G″). We found some endoderm cells in the node (Figure 3.3E, G, stars) and the node did not form the typical teardrop shape in *Sox17*$^{-/-}$ embryos (Figure 3.3E-G″). Scanning electron microscopy also confirmed normal cilia formation as well as the presence of isolated endoderm cells (arrowheads) in the node and segregated groups of ciliated cells (arrow) posterior to the node in *Sox17*$^{-/-}$ embryos (Figure 3.3I, J). Since ectopic endoderm cells in the node of *Sox17*$^{-/-}$ embryos might prevent the differentiation

Figure 3.3. Node morphogenesis is partially defective, however the node is able to direct LR asymmetry in the *Sox17* mutants. Immunohistochemistry of anti-γ-tubulin for basal bodies (magenta in A, B, E, F), anti-acetylated tubulin (Actu) for cilia (green in B, F; magenta in C, G), anti-ZO1 for tight junction (green in A, C, E, G). *Nodal* mRNA (D, H) in the *Sox17*$^{+/-}$ (A-D) and *Sox17*$^{-/-}$ embryos (E-H) at the 1- to 2-somite stage (A-C, E-G) or the headfold sage (D, H). Note that in the *Sox17*$^{-/-}$ embryos, some endoderm cells have not migrated out from the node region (*), resulting in the posterior elongation of the node. The shape of nodes is highlighted with red color (C", D"). An arrow indicates isolated node region at the posterior (D"). The *Nodal* expression in the node also showed posterior elongation of the node in the *Sox17*$^{-/-}$ embryo (H, arrow). (H-I') Node structures and cilia formation were examined by scanning electron microscopy. Positions of H' and I' are indicated by rectangles in H and I. Note that two large endoderm cells remained in the node (red stars) and ectopic ciliated cells (arrow) were observed in the separated region from the *Sox17*$^{-/-}$ node. (J and K) nodal flow in *Sox17*$^{+/-}$ and *Sox17*$^{-/-}$ nodes. Each line represents tracings of bead movement over the node surface from the movies (see supplementary movies 1 and 2). The black lines outline the boundary of the node. (L-Q) Both *Sox17*$^{+/-}$ *and Sox17*$^{-/-}$ perinode cells expressed *Nodal* (L, O), *L-Plunc* (M, P), and *Cerl2* (N, Q) asymmetrically. Ectopic *Cerl2* expression in the posterior to the *Sox17* $^{-/-}$ node was observed in the *Sox17*$^{-/-}$ embryo (arrow).


γ-Tubulin/ZO1
γ-Tubulin/Actu
Actu/ZO1
Sox17+/-
Sox17-/-
A
B
C
C''
A'
B'
C'
D
Nodal
50 μm
500 μm
E
F
G
G''
E'
F'
G'
H
Nodal
P
A
I
I'
J
J'
K
L
R
P
A
L
50 μm
5 μm
Nodal
L-Plunc
Cerl2
M
N
O
L
R
500μm
P
Q
R

of node precursors into mature ciliated cells, the number of node cells was examined by γ-tubulin and ZO1 (Tjp1 – Mouse Genome Informatics) staining (Figure 3.3A, E). No statistically significant difference was seen between *Sox17*$^{-/-}$ and control nodes [299±5.5 (*n*=5) and 302±5.9 (*n*=5), respectively; *P*=0.76, Student's *t*-test].

As cell number and cilia formation in the node appeared normal in *Sox17*$^{-/-}$ embryos, we next determined whether the abnormal shape of the node might affect the direction and strength of the nodal flow that provides a bias to determine the initial LR asymmetry. When the flow was visualized with fluorescent beads, the flow rate and direction were similar in *Sox17*$^{-/-}$ and control embryos; however, the smooth pattern of the flow was slightly affected by the presence of the remaining endoderm cells in the *Sox17*$^{-/-}$ nodes (Figure 3.3K, L; supplementary material movies 1, 2; *n*=9 and *n*=7 for control and mutant embryos, respectively). Importantly, a significant leftward flow developed in all *Sox17*$^{-/-}$ embryos examined.

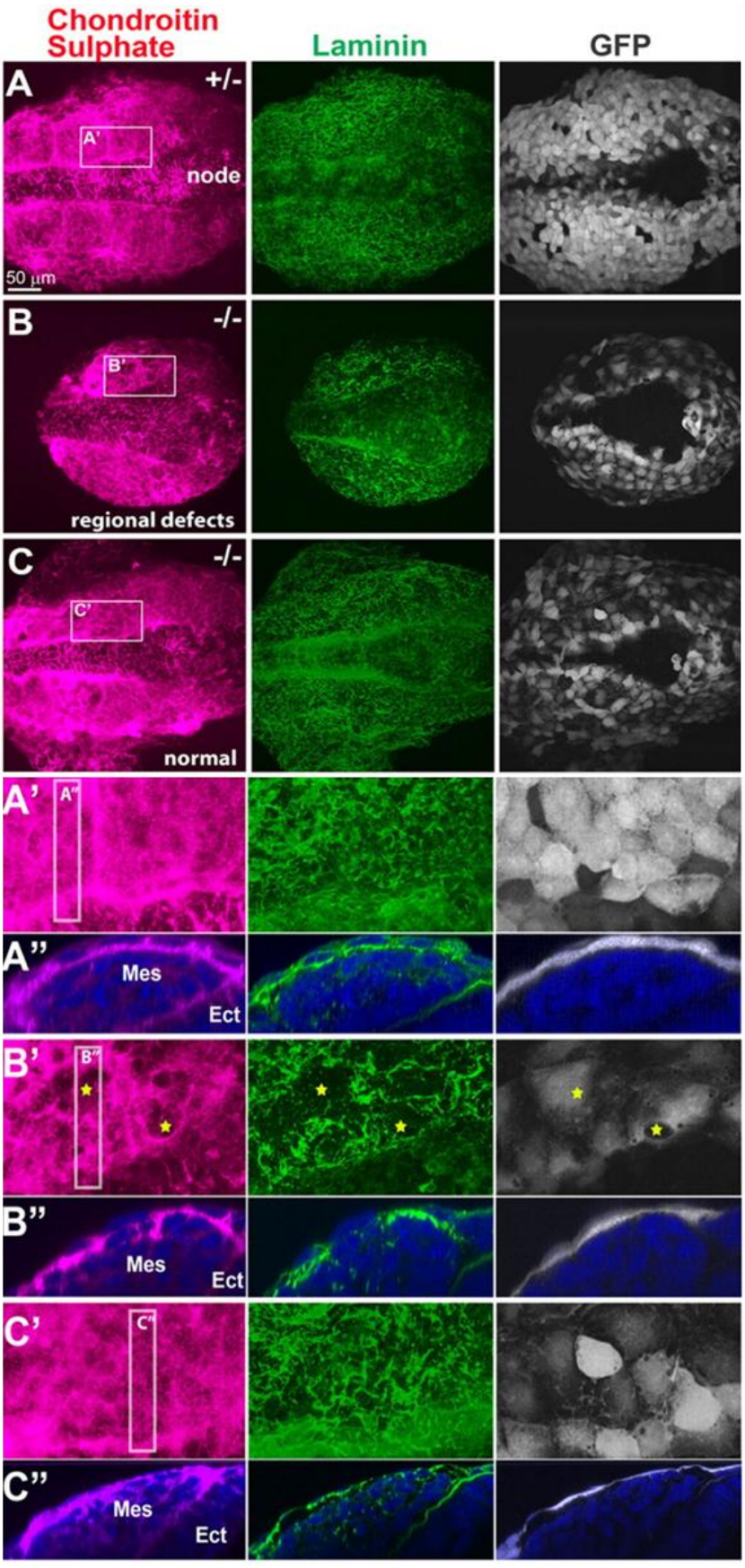
To evaluate the outcome of nodal flow on the generation of LR polarity across the node, we examined the asymmetric expression of the perinodal genes *Nodal, L-Plunc* (*Bpifb1* – Mouse Genome Informatics) and *Cerl2* (*Dand5* –Mouse Genome Informatics) (Hou et al., 2004; Pearce et al., 1999). All three genes were asymmetrically expressed in the node in most *Sox17*$^{-/-}$ embryos (*Nodal*, 7/11 (64%); *L-Plunc*, 9/11 (82%); *Cerl2*, 10/11 (91%); Figure 3.3P-R), similar to control embryos (*Nodal*, 15/22 (68%); *L-Plunc*, 11/11 (100%); *Cerl2*, 9/9 (100%); Figure 3.3M-O). These data suggested that in *Sox17*$^{-/-}$ embryos the nodal flow is strong enough to generate LR asymmetry in the node despite some abnormal features of the node.

Partial defects in proteoglycan distribution in *Sox17*$^{-/-}$ embryos

Finally, we examined the possibility that disrupted transfer of the LR signals from the node to the left LPM accounts for the LR defects observed in *Sox17*$^{-/-}$ embryos. Previously, we reported that proteoglycans with glucosaminoglycan moieties such as chondroitin sulfate and heparan sulfate play important roles in transferring the LR signals from the node to the LPM (Oki et al., 2007). Chondroitin sulfate is distributed at the interface between endoderm and mesoderm in control embryos, and was shown to directly interact with NODAL proteins (Oki et al., 2007). We performed immunohistochemistry (IHC) of chondroitin sulfate and laminin at 2- to 4-somite stage to study the effect of *Sox17* mutation on proteoglycan distribution and ECM development (Figure 3.4A-C). We found that 7 of 17 (41%) *Sox17*$^{-/-}$ embryos showed regional defects in chondroitin sulfate distribution concomitant with laminin matrix disorganization (Figure 3.4B-B″), whereas the remaining 10 of 17 (59%) *Sox17*$^{-/-}$ embryos showed an almost normal distribution of ECM (Figure 3.4C-C″; control embryos, n=6). These defects in chondroitin sulfate distribution in the ECM might cause reduced LR signal transfer from the node; however, the loss of *Nodal* expression in the LPM in 90% of *Sox17*$^{-/-}$ embryos cannot be accounted for by the reduced chondroitin sulfate distribution and defective ECM composition seen in only 41% of these embryos.

Since paraxial mesoderm lies between the node and the LPM, it could conceivably play a role in mediating transfer of the LR signal from the node to the LPM. To test this, we determined whether the paraxial mesoderm develops normally in *Sox17* mutants. In *Sox17*$^{-/-}$ embryos, both *Meox1*, which regulates somitogenesis (supplementary material), and *Raldh2* (*Aldh1a2* – Mouse Genome Informatics), a key

Figure 3.4 Defects in Proteoglycan and ECM distribution are not the primary cause of LR abnormalities in *Sox17* mutants. (A-C) Coimmunohistochemistry of chondriotin sulphate, laminin and GFP in $Sox17^{GFP/+}$ and $Sox17^{GFP/GFP}$ embryos at the 3- to 4-somite stage. The proteoglycan chondroitin sulphate and the ECM component laminin are distributed uniformly in $Sox17^{GFP/+}$ embryos (A-A"), whereas in $Sox17^{GFP/GFP}$ embryos, there are either regional defects (B-B", n = 7/17) or almost normal (C-C", n = 10/17) organization of chondriotin sulphate and laminin. Yellow stars in B' indicate localized regions of reduced chondroitin sulphate and laminin distribution. Ect, ectoderm; Mes, mesoderm.


Chondroitin Sulphate
Laminin
GFP
A
+/-
A'
node
50 μm
B
-/-
B'
regional defects
C
-/-
C'
normal
A'
A''
A''
Mes
Ect
B'
B''
B''
Mes
Ect
C'
C''
C''
Mes
Ect

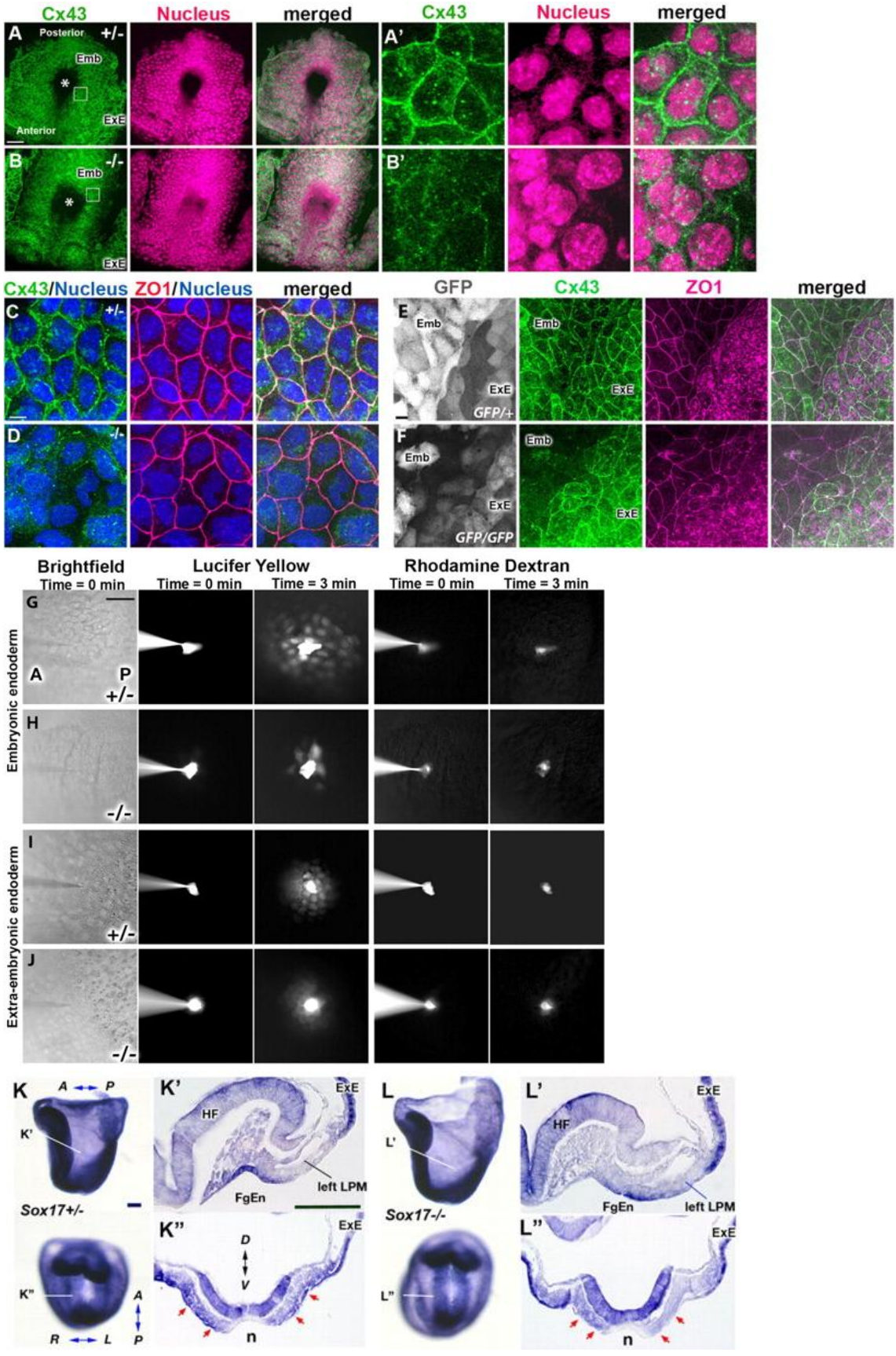
gene for retinoic acid synthesis that coordinates symmetric somitogenesis and LR asymmetry (Vermot and Pourquie, 2005; supplementary material), were expressed normally, suggesting that paraxial mesoderm is normal in *Sox17*$^{-/-}$ embryos.

Reduced gap junction function in *Sox17* mutant epithelial cells

After nodal flow is established, intracellular upregulation of Ca^{2+} flux is observed in endoderm cells immediately left of the node (Hadjantonakis et al., 2008; McGrath et al., 2003; Tanaka et al., 2005). This suggests that Ca^{2+} flux might have an important role in signal transfer of LR determination from the node to the LPM (Mercola, 2003). Since the relay of Ca^{2+} flux is mediated by gap junctional transport of small molecules such as IP3 or Ca^{2+} itself, we examined the expression of a gap junction protein, connexin43 (Cx43; Gja1 – Mouse Genome Informatics), by whole-mount IHC, which had not been described previously for the time when LR asymmetry is established. In control embryos, we found that CX43 was localized in the cell membrane in both extraembryonic and embryonic endoderm, but not in the node including perinode cells (Figure 3.5A, E; supplementary material). In *Sox17*$^{-/-}$ embryos, CX43 was localized in the cell membrane of extraembryonic endoderm but was greatly reduced in the endoderm cells in the embryonic region (Figure 3.5B, F). We also observed that the localization of another connexin, CX40 (Gja5 –Mouse Genome Informatics), on the cell membrane was attenuated in the endoderm of *Sox17*$^{-/-}$ versus control embryos (data not shown).

We next assessed the level of *Cx43* mRNA and found that *Cx43* expression was reduced in endoderm cells near the node in *Sox17*$^{-/-}$ embryos (Figure 3.5L″, red arrows) as compared with control embryos (Figure 3.5K″), whereas *Cx43* mRNA was expressed normally in both the foregut and extraembryonic endoderm of *Sox17*$^{-/-}$ embryos

Figure 3.5 Defective Cx43 localization and gap junctional transport in *Sox17* $^{-/-}$ endoderm cells. Cx43 (green in A-F), nuclei (magenta in A, B, blue in C, D), ZO1 (magenta in C-F), GFP (white in E, F), stars denote the node (A, B). In control embryos, CX43 protein is normally localized in cell membranes of extraembryonic (ExE) and embryonic (Emb) endoderm cells (A-A', E, F). In *Sox17*$^{-/-}$ embryos at 3-somite stage, localization to cell membranes is disrupted only in embryonic endoderm cells but not in the extraembryonic endoderm cells (B-B', E, F). A' and B' represent enlarged embryonic region of white rectangles in A and B, respectively. In contrast, ZO1 was localized on the cell boundaries in both *Sox17*$^{-/-}$ and *Sox17*$^{+/-}$ endoderm cells in the embryonic (C-F) and extraembryonic regions (E, F). A', B', C, D show embryonic endoderm cells. Scale bars: 50μm in A, B, 5μm in C, D, and 10μm in E, F. Gap junctional transport was examined by iontophoresis of fluorescent dyes. A mixture of Lucifer Yellow and Rhodamine dextran (MW 10 kD) was injected into endoderm cells in the embryonic region (G, H) and extraembryonic endoderm (I, J) cells. Three minutes after the injection, Lucifer Yellow dye was transferred to the surrounding cells in the embryonic region in *Sox17*$^{+/-}$ embryos (G) as well as extraembryonic endoderm cells in both *Sox17*$^{+/-}$ and *Sox17*$^{-/-}$ embryos (I, J) but weakly transferred in *Sox17*$^{-/-}$ embryos (H). Scale bar: 50 μm. (K-L) Patterns of *Cx43* mRNA in *Sox17*$^{+/-}$ (K-K") and *Sox17*$^{-/-}$ embryos (L-L") at 2-somite stage. Position of sections (K', K", L', L") are indicated by white bars in K and L. Red arrows show *Cx43* expression in endoderm cells near the node (K", L"). Note that no CX43 expression was observed in the ventral side of the node (n). Emb, embryonic endoderm ExE, extraembryonic endoderm; FgEn, foregut endoderm; HF, headfold. Scale bars: 100 μm.


Cx43
Nucleus
merged
Posterior
+/-
Emb
ExE
Anterior
-/-
A
A'
B
B'
Cx43/Nucleus
ZO1/Nucleus
C
D
GFP
ZO1
E
F
GFP/+
GFP/GFP
Brightfield
Lucifer Yellow
Rhodamine Dextran
Time = 0 min
Time = 3 min
Embryonic endoderm
Extra-embryonic endoderm
G
H
I
J
A
P
K
K'
K"
L
L'
L"
HF
FgEn
left LPM
Sox17+/-
Sox17-/-
D
V
n

(Figure 3.5K-L″).

Next, gap junction function was tested by iontophoresis of a gap junction permeable dye, Lucifer Yellow. Three minutes after dye injection the Lucifer Yellow spread from the injected cell into neighboring cells of the embryonic endoderm (*n*=10, average 33.1 cells and 4.5 cell diameters; Figure 3.5G) or extraembryonic endoderm in control embryos (Figure 3.5I), whereas the impermeable dye Rhodamine dextran was retained in the injected cell. In *Sox17*$^{-/-}$ embryos, the Lucifer Yellow dye, but not Rhodamine dextran, spread into neighboring cells of the extraembryonic endoderm (Figure 3.5J); however, in embryonic endoderm, both injected dyes stayed in the injected cell (*n*=6/23 injections) or the Lucifer Yellow expanded into significantly fewer cells (*n*=17/23, average 6.6 cells and 1.4 cell diameters; $P<0.001$, Student's *t*-test) (Figure 3.5H) than in the control embryo.

In conjunction with the reduction of *Cx43* mRNA and in the membrane localization of connexin proteins, these data clearly indicate that *Sox17*$^{-/-}$ endoderm has defective gap junction communication, which is likely to be the major cause of defective LR determination in *Sox17*$^{-/-}$ embryos.

Sox17 mutants exhibit defects in the formation of epithelial structures in endoderm cells

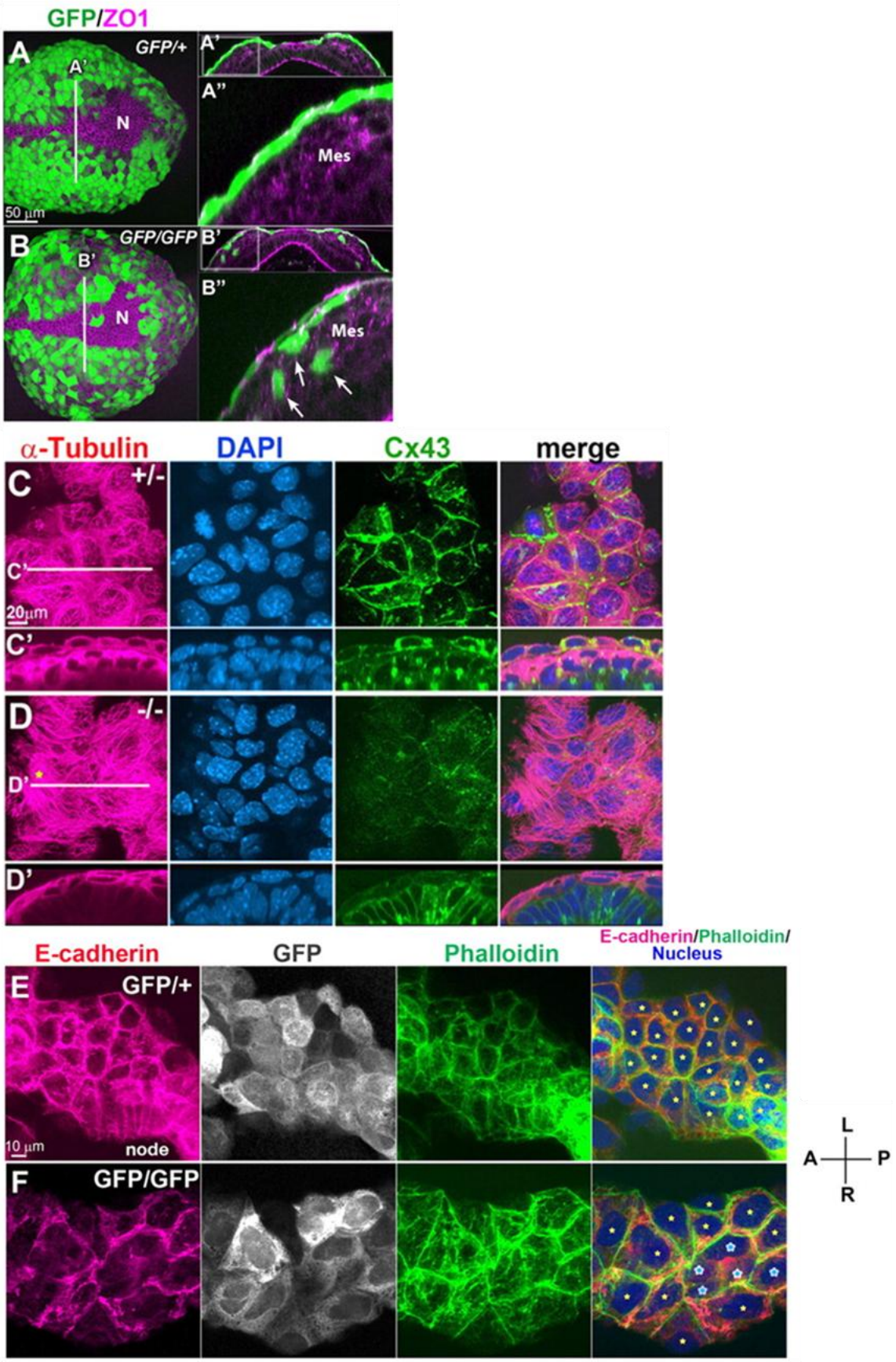
Why was Cx43 not distributed in the cell membrane in *Sox17* mutants? We first tested the possibility that the mislocalization of connexin proteins might be due to a lack of cell-cell contacts. The tight junction component ZO1 was localized normally on the cell membrane of *Sox17*$^{-/-}$ endoderm cells, similar to control endoderm (Figure 3.5C, D); this suggests that *Sox17* mutant endoderm develops an epithelial structure with tight

junctions.

We further evaluated the epithelial organization of *Sox17* mutant endoderm that lies in close proximity to the node at stages corresponding to LR signal transfer from the node to the LPM (1- to 3-somite stage). We made use of *Sox17GFP* knock-in mice, in which a *GFP* reporter cassette replaces the coding region of the *Sox17* locus, resulting in null mutation and placing GFP expression under the control of the endogenous transcriptional regulation of *Sox17* (Kim et al., 2007).

In $Sox17^{GFP/+}$ embryos at 2-somite stage, whole-mount IHC of GFP and the tight junction component ZO1 showed GFP expression in the tightly packed endoderm cells as a contiguous epithelial layer overlying mesoderm cells, encompassing the entire embryonic region except the midline and the node (Figure 3.6A). Endoderm cells were attached to each other over a relatively large area of contact (Figure 3.6A′, A″). In $Sox17^{GFP/GFP}$ embryos, although the endoderm layer covered the entire embryo in a manner similar to that of control endoderm, the endoderm cells constituting the endoderm layer were thinner and larger than those of the control embryo, with a greatly reduced area of contact between cells (Figure 3.6B-B″). Unexpectedly, some GFP-positive cells in $Sox17^{GFP/GFP}$ embryos were found interspersed among the underlying mesoderm cells on either side of the midline (Figure 3.6B′, B″). We hypothesized that these misplaced cells might be delaminated from the outer endoderm layer, possibly owing to the loss of epithelial cell polarity, or that these cells might be derived from endodermal cells that have failed to epithelialize after migrating out from the primitive streak. From these observations, we hypothesized that SOX17 is required for uniform structural and functional epithelial integrity in endoderm cells.

Figure 3.6 Loss of *Sox17* results in defects in epithelial polarity and disorganized cellular adhesion. GFP and ZO1 immunohistochemistry in $Sox17^{GFP/+}$ and $Sox17^{GFP/GFP}$ embryos at the 3-somite stage (A, B). Optical transverse sections from the reconstructed confocal images showed a compact epithelial layer of definitive endoderm cells in $Sox17^{GFP/+}$ embryos (A', A"), whereas in $Sox17^{GFP/GFP}$ embryos the epithelial layer was uneven and a portion of GFP-positive cell population was found interspersed among the mesoderm cells (m; B', B"). (C, D) Coimmunohistochemistry of α-tubulin, Cx43 with nuclear staining in $Sox17^{+/-}$ and $Sox17^{-/-}$ embryos at the 3-somite stage. Microtubules labeled with α-tubulin showed a parallel arrangement in $Sox17^{-/-}$ endoderm (star, D) compared to the uniformly distributed localization in $Sox17^{+/-}$ embryos, accompanied by a reduction in the membrane localization of CX43 (optical transverse sections in C', D'). (E, F) E-cadherin, GFP and actin distribution in embryos. Notice the disorganized localization of E-cadherin in the $Sox17^{GFP/GFP}$ embryos (F) compared to the control embryos (E), suggesting weaker adherens junction formation. Stars indicate individual cells, and particularly, the blue stars indicate cells with highly reduced E-cadherin distribution in the cell boundary. Mes, mesoderm; N, node.


GFP/ZO1
A
GFP/+
A'
N
A"
Mes
50 μm
B
GFP/GFP
B'
N
B"
Mes
α-Tubulin
DAPI
Cx43
merge
C
+/-
C'
20μm
C'
D
-/-
D'
D'
E-cadherin
GFP
Phalloidin
E-cadherin/Phalloidin/
Nucleus
E
GFP/+
10 μm
node
F
GFP/GFP
L
A
P
R

To determine whether the abnormal endoderm development in *Sox17* mutants affects the membrane localization of gap junctions, we compared microtubule organization and CX43 localization by co-IHC in control and *Sox17* mutant endoderm cells (Shaw et al., 2007; Thomas et al., 2005). In control embryos, microtubules labeled by α-tubulin were distributed in a radial fashion and along the cell membrane (Figure 3.6C, C′), whereas in *Sox17* mutant embryos, which showed severely reduced CX43 membrane distribution, α-tubulin was primarily present in a fibroblast-like parallel arrangement in the cytoplasmic region and did not show clear accumulation at the cell boundaries (Figure 3.6D, D′; Bre et al., 1990).

Adherens junctional complexes comprising E-cadherin and β-catenin constitute the main source of mechanical adhesion between adjacent epithelial cells (Baum and Georgiou, 2011). In endoderm cells neighboring the node of control embryos at 3-somite stage, E-cadherin was evenly localized in the basolateral membrane (Figure 3.6E), whereas in *Sox17*$^{-/-}$ embryos E-cadherin distribution was reduced in most cells and was absent from some endoderm cells (Figure 3.6F, blue stars). Labeling of actin filaments with phalloidin also showed an uneven localization of actin fibers at the cell boundary in endoderm cells of *Sox17* mutants (Figure 3.6F). Similar patterns were observed for β-catenin, which was not localized at cell boundaries in some endoderm cells (data not shown). These defects suggest weak intercellular adhesion between endoderm cells, even though ZO1 is localized normally on the cell membrane of *Sox17* mutant endoderm cells (Figure 3.6D).

Abnormal cytoskeletal assembly together with the incomplete formation of adherens junctions demonstrate defects in the epithelial features of *Sox17* mutant

endoderm. These defects could result in the loss of gap junction communication, either by lowering the levels of *Cx43* transcription or by reducing the localization of CX43 to the cell membrane. This might cause defects in transferring LR signals from the node to the LPM.

Discussion

All tissues that have previously been reported to play a role in LR determination are mesodermal derivatives, such as the node, LPM, notochord and somites. Here, we report a novel role for endoderm cells in transferring LR signal information from the node to the LPM.

Sox17 mutants develop endoderm-specific defects and lose signal transfer

Sox17$^{-/-}$ endoderm cells exhibit epithelial disorganization. Despite this, the tight junction component ZO1 is still expressed normally, suggesting that the endoderm in the mutant maintains partial epithelium formation. Endoderm constitutes the outermost layer of the early embryo and plays an important role in homeostasis of the embryo by forming a tight junction barrier to the external environment. It should be noted that mesodermal and ectodermal, but not endodermal, derivatives form normally in *Sox17* mutants (Figure 3.2; data not shown).

The overall shape of the node in *Sox17*$^{-/-}$ embryos is affected by the presence of isolated endoderm cells that fail to clear from the node region; however, LR determination at the node seems to develop normally, as evidenced by the relatively normal nodal flow and the asymmetric expression of perinodal genes. Similarly, the LPM

in *Sox17* mutants also develops normally, with the potential to express and propagate *Nodal,* as shown when triggering NODAL proteins were exogenously supplied directly into the LPM. Therefore, the most likely cause of the absent or reduced LR gene expression observed in *Sox17* mutants is a failure to transfer the LR signal from the node to the LPM.

Conserved gap junction function in establishing LR asymmetry in mammals

In this study we found that the localization of the gap junction molecules CX43 and CX40 to the cell membrane of the embryonic endoderm was greatly impaired in *Sox17* mutants. Physiological assays of gap junction communication indicated that it was dysfunctional in mutant endoderm. These findings suggest the importance of gap junctions in endoderm for LR signal transfer. In addition to our data, it was recently reported that a pharmacological inhibitor of gap junction communication, 18-α-glycyrrhetinic acid, could inhibit *Nodal* expression in the left LPM of wildtype embryos (Viotti et al., 2012). Although these inhibitor experiments did not specifically identify the endoderm as the tissue responsible for the gap junction communication in LR determination, when combined with our findings from genetic analyses of *Sox17* mutants, the data support the idea that gap junctions in the endoderm are necessary for signal transfer from the node to the LPM in the mouse embryo.

Gap junctions have been reported to function in LR determination in several vertebrate models (Levin and Mercola, 1998; Levin and Mercola, 1999). In rabbits, gap junctions are used to prevent signal transfer from the node to the right LPM in cooperation with Fgf8 signaling (Feistel and Blum, 2008). Although, in this case, the

tissues responsible for the gap junction communication were not clearly identified, *Cx43* is expressed in the endoderm as well as in the mesoderm and ectoderm of the rabbit embryo, similar to the mouse (Figure 3.5; supplementary material). These data suggest that a role for endoderm cell gap junctions in mediating signals from the node to the LPM is conserved in mammals.

Impaired epithelial integrity in the *Sox17* mutant endoderm and possible roles of endoderm in signal transfer from the node to the LPM

This is the first report that demonstrates the role of SOX17 in the development of the epithelial characteristics of endoderm at the cellular level. We examined the distribution of cell adhesion and cytoskeleton components in *Sox17*$^{-/-}$ and control embryos and found that endoderm in *Sox17* mutants exhibits compromised epithelial integrity, in which the localization of E-cadherin/β-catenin on the cell membrane is disturbed and microtubules and actin filaments show aberrant arrangement. These findings strongly suggest that the severe impairment of gap junction communication in mutant endoderm is a consequence of the failure to properly localize connexin proteins on cell boundaries due to impaired epithelial polarity. Thus, epithelial maturity of endoderm cells near the node is essential for functional gap junction communication among endoderm cells and is involved in transferring signals from the node to the LPM in LR determination.

How do endoderm cells near the node mediate signal transfer from the node to the LPM? In accordance with the evidence of higher Ca^{2+} flux in the left endoderm near the node and the fact that the inhibition of gap junction communication results in defects in

LR determination (Viotti et al., 2012), one explanation is that endoderm directly transfers second messengers such as Ca^{2+} through gap junctions from the node to the LPM to induce *Nodal* expression in the LPM. However, the higher Ca^{2+} flux in the left endoderm is observed only near the node, suggesting short-range signal transduction. Therefore, it is unlikely that Ca^{2+} itself signals over a long distance to transmit the LR information from the node to the LPM.

In addition to several studies showing that NODAL proteins act over a long range by diffusion (Chen and Schier, 2001; Constam, 2009; Le Good et al., 2005), a recent report strongly suggests that NODAL proteins produced in the perinode travel a long distance to induce *Nodal* expression in the left LPM (Kawasumi et al., 2011). Given these data, endoderm might play a role in supporting long-range NODAL movement. The long-range travel of NODAL proteins appears to require extracellular matrix (ECM) components such as chondroitin sulfate (Oki et al., 2007). In *Sox17* mutants, the ECM components chondroitin sulfate and laminin were formed relatively normally; however, it remains possible that there are other, as yet unidentified, ECM components that are required for NODAL movement and are affected in the mutant endoderm because of the incomplete epithelial polarity. An alternative possibility is that endoderm plays a role in the modification of NODAL proteins. The range of travel and activities of NODAL are regulated by processing of prepro-NODAL proteins (Ben-Haim et al., 2006), N-glycosylation (Le Good et al., 2005) and dimer formation with NODAL itself or another TGFβ member, GDF1 (Tanaka et al., 2007). Since *Sox17*-expressing endoderm is tightly attached to the perinode where NODAL is produced, endoderm might assist in these modifications through cell-cell communication with perinode cells.

Ectopic expression of *Nodal* in endoderm cells of *Sox17*$^{-/-}$ embryos

We found that the foregut endoderm of *Sox17*$^{-/-}$ embryos expresses ectopic *Nodal* at early somite stages (Figure 3.1D, D′, G). NODAL is an upstream regulatory factor that induces endoderm formation during gastrulation (Tremblay et al., 2000) but the expression is immediately shut down in differentiated endoderm (Collignon et al., 1996). These observations suggest that SOX17 might be directly or indirectly required to turn off *Nodal* expression in differentiating endoderm. If this were the case, we would predict that continued expression of *Nodal* might also contribute to the failure of endoderm formation in *Sox17*$^{-/-}$ embryos. Further analysis will be required to test this directly.

How does loss of function of *Sox17* affect LR signal transfer?

We have shown that *Sox17* mutants possess a narrower node compared to normal embryos, although the number of cilia is similar and nodal flow occurs in the normal leftward orientation. Left right asymmetries across the node are also preserved as evidenced by *Nodal, L-Plunc* and *Cerl-2* expression in the perinode in *Sox17* mutants. These data indicate that the smaller node in the mutants does not affect the core mechanisms of the LR symmetry breaking event in the node in *Sox17* mutants. However, the structural abnormalities of the node suggest that either the magnitude of the LR signal generated on the left side of the node or the subsequent signal transfer process may be indirectly affected in *Sox17* mutants. Closer examination of the node by scanning electron microscopy and ZO1 localization in *Sox17* mutants revealed the presence of isolated endoderm cells within the node pit. Furthermore, the organization of the perinode

cells into discrete bilateral structures appeared to be affected in the mutant embryos. Whole mount *in situ* hybridization of *Nodal* in *Sox17* control and mutant embryos at the 4-somite stage revealed that the perinode structure was disjointed and small groups of isolated perinode cells were formed in the mutant embryos (Figure 3.7A-C). The abnormal perinode organization begins at the presomite stage in *Sox17* mutants as indicated by the *Nodal-5'-LacZ* signal at the 0-somite stage (Figure 3.7D-F). At this stage, there were either regional accumulation of perinode cells or discontinuity of the bilateral perinode structure in the mutant embryos. These results suggest that the differentiation of perinode cells was not affected, however, *Sox17* may play a role in the coherent assembly of the perinode structure. Analysis of *Sox17* expression pattern had previously revealed robust SOX17-GFP localization in the endoderm at the margin of the node overlying the perinode cells in *Sox17*$^{GFP/+}$ embryos at the 3- to 4-somite stage (supplementary material).

The node, including the perinode cells, is formed from the anterior most tip of the primitive streak that is in direct contact with the endoderm cells before overt node formation. During node morphogenesis, the endoderm cells are cleared to allow the node cells to emerge into the exterior and form the characteristic oval shaped structure (Sulik et al., 1994). The presence of isolated endoderm cells within the node in *Sox17* mutants at the 0- to 1-somite stage suggests that the clearing or movement of endoderm cells away from the underlying premature node may be affected in the mutant embryos (Figure 3.7). It is likely that the endoderm migration defects may also affect the aggregation of perinode cells into the distinct horseshoe-shaped structure in the *Sox17* mutants. Live imaging of transgenically labeled endoderm cells will be useful to investigate how

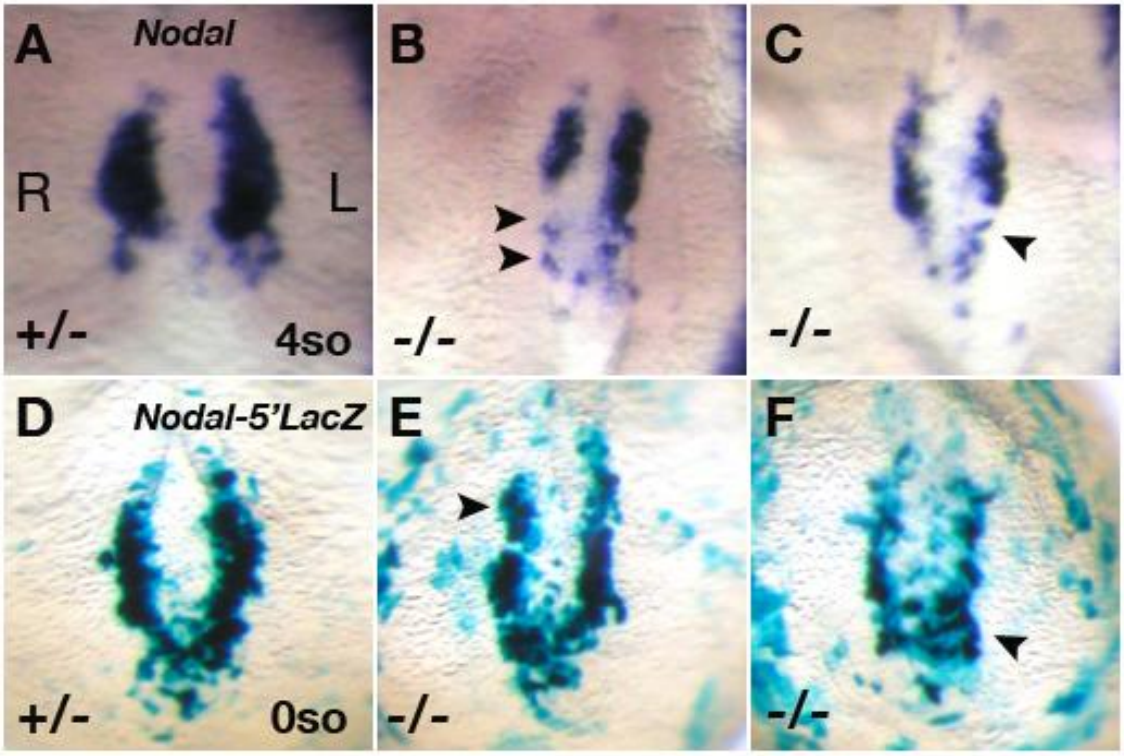


Figure 3.7 Abnormal organization of the perinode in *Sox17* mutants. (A-C) *Nodal* is expressed asymmetrically in the perinode in *Sox17*+/- embryos at 4-somite stage (A), however, the perinode appears disorganized in *Sox17*-/- mutants (B, C). The arrowheads indicate segregated perinode cells expressing *Nodal*, revealing the discontinuous arrangement of perinode cells. (D-F) At 0-somite stage, *Nodal-5'LacZ* transgene is active in the horseshoe shaped perinode in *Sox17*+/- embryos and shows subtle LR asymmetry (D), whereas in *Sox17*-/- embryos, several isolated groups of perinode expressing the transgene are observed (arrowheads E, F).

endoderm migrates over the developing node in control and mutant embryos.

How does abnormal perinode organization contribute to defects in LR determination in *Sox17* mutants? Previous studies by Saijoh and colleagues have clearly shown the requirement of *Nodal*, produced in the node, for the expression of *Nodal* in the left LPM. This is demonstrated by the rescue of *Nodal* expression in the LPM by perinode specific expression of transgenic *Nodal* in embryos homozygous for a hypomorphic *Nodal* allele (Saijoh et al., 2003). Thus, it is likely that the levels of *Nodal*

expression in the perinode may be important for the transmission of LR signal information from the node to the LPM. In *Sox17* mutants, the symmetry breaking across the node occurs in the leftward orientation as evidenced by the left-oriented nodal flow and the asymmetric gene expression patterns in the perinode cells (Saund et al., 2012). However, the abnormal organization of the perinode indicated by the disordered *Nodal* expression in the mutant embryos may result in lower levels or delayed accumulation of LR signal information on the left side of the node relative to the control embryos. In support of this possibility, we had previously observed that the *Nodal*, *Lefty2* and *Pitx2* expression in the left LPM showed a delayed onset and restricted pattern in the *Sox17* mutants (Chapter 2). Cross signaling between the perinode cells and the endoderm may be important to activate the NODAL protein complex within the perinode. Thus, the defects in endoderm differentiation in *Sox17* mutants not only result in irregularities in perinode structure, but may also affect processing of NODAL that is necessary for the transfer of the asymmetric signal from the node to the LPM.

Following the breaking of LR symmetry at the node, the next step is the signal transfer process from the node to the LPM. Oki et al., have suggested that the LR signal takes an internal route that involves interaction of free NODAL protein with sulfated glycosaminoglycans (GAGs) that are enriched at the basement membrane between the endoderm and the mesoderm (Oki et al., 2007). The authors reported that NODAL interacts with chondroitin sulfate (CS) *in vitro* and *Nodal* expression in the LPM was absent in embryos cultured with an inhibitor of CS biosynthesis. This suggests that the basement membrane may provide an alternate route for the LR signal transfer. Previous studies have implicated sulfated GAGs in LR asymmetry, using similar inhibition studies

in frog embryos that resulted in abrogation of heart looping and were found to be specifically required immediately before the initiation of the asymmetric *Xnr-1* in the LPM (Yost, 1990). There is evidence that the sulfated proteoglycans distributed in the ECM can promote travel of the TGF ligand, NODAL, over long distances in studies involving animal cap assays in xenopus (Marjoram et al., 2011).

We, thus, examined the spatial distribution of ECM component laminin and the sulfated proteoglycan, chondriotin sulfate (CS) by whole mount immunohistochemistry in *Sox17* mutant embryos and analyzed the region between the node and the LPM (Figure 3.8). To explore how the epithelial polarity defects in endoderm may impact laminin and CS localization, we employed the $Sox17^{GFP}$ allele, which labels the surface endoderm cells with GFP. In $Sox17^{GFP/+}$ control embryos, both laminin and CS proteins are uniformly and robustly localized in the basement membrane between the endoderm layer and the underlying mesoderm cells, extending from the perinode cells to the LPM (Figure 3.8A-C). Additionally, CS is also found interspersed between the mesoderm cells, however, the distribution is weaker than the basement membrane between the endoderm and mesoderm. These data suggest that the sulfated GAG bound diffusion of the NODAL morphogen ligand along the basement membrane is the primary route of long distance movement from the node to the LPM in normal embryos, following secretion of NODAL from the perinode. In contrast to the uniform pattern of laminin and CS distribution in the basement membrane underneath the endoderm layer, the $Sox17^{GFP/GFP}$ null mutant embryos showed a patchy distribution, where large sites were deficient in both laminin and CS localization (Figure 3.8A'-C'). The mutant endoderm formed a thinner layer,

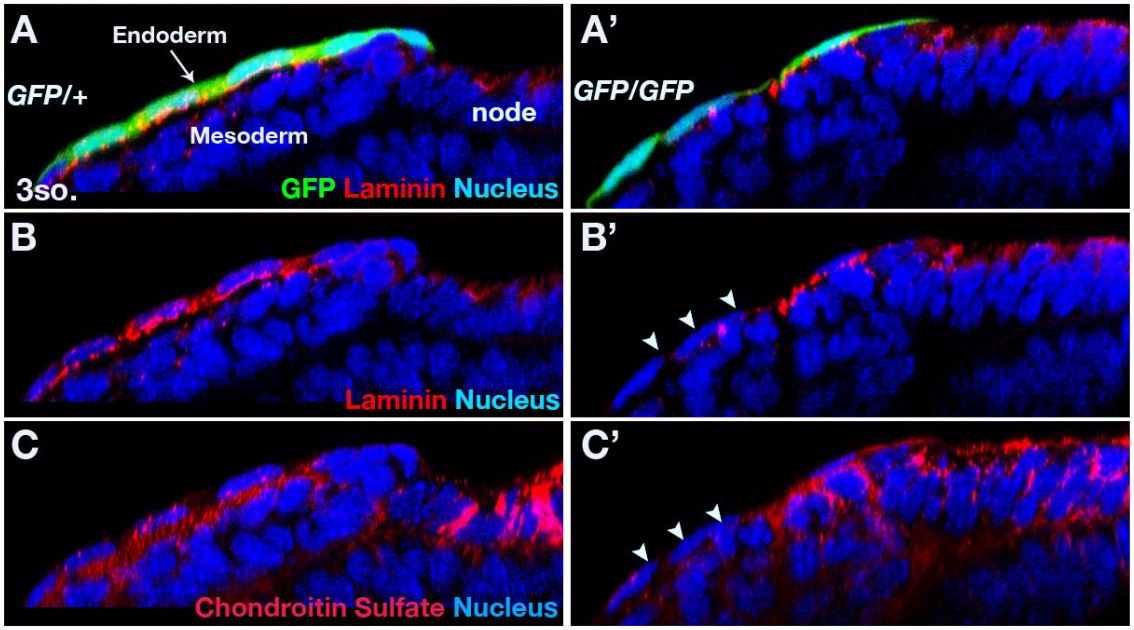


Figure 3.8 Laminin and chondroitin sulfate distribution is discontinuous in the basement membrane in *Sox17* mutants. (A-C) Transverse section of z-stacked confocal images of a *Sox17*$^{GFP/+}$embryo at 3-somite stage showing the endoderm layer labeled with GFP (green), laminin (red, A, B) and chondriotin sulfate (red, C) distribution in the basement membrane. Mesoderm and node cells are indicated by nuclear staining by DAPI (blue). (A'-C') In *Sox17*$^{GFP/GFP}$ embryos, the basement membrane appears discontinuous and shows reduced localization of Laminin and chondroitin sulfate (arrowheads) at several sites between the endoderm and mesoderm layers.

with uneven thickness compared to control embryos, suggesting that the abnormal epithelial morphology likely affects the matrix-like organization of the basement membrane.

Our studies had previously also shown that the several endoderm cells expressing GFP were found delaminated within the mesoderm layer in the mutant embryos, which might also contribute to the noncontinuity of the basement membrane. Examination of the different types of integrin receptors in mutant embryos may be useful to test whether the loss of basal polarity in endoderm cells directly affects interaction with the basement membrane. Although the association of NODAL with CS contained in the ECM remains

to be shown *in vivo*, these observations suggest that the irregular and discontinuity in the ECM organization may negatively affect the long distance transport of NODAL, required to activate Nodal signaling in the LPM. In about half the *Sox17* mutant embryos, we observed partial activation of Nodal signaling in the left LPM, indicated by *Lefty2* and *Pitx2* expression. This suggests either both delayed and or insufficient levels of NODAL protein reaching the LPM. Taken together, it is likely that this result may be a cumulative effect of the irregular organization of the perinode and ECM in the basement membrane in *Sox17* mutants, affecting both the initial release and subsequent transfer of the asymmetric signal to the LPM, respectively.

In summary, we have identified a novel and important role of endoderm cells in signal transfer from the node to the LPM during the establishment of LR asymmetry in mice. The detailed molecular mechanisms of how endoderm participates in the signal transfer remain to be examined in future studies. However, our present studies detailing *Sox17* mutants will help further investigations and will significantly extend the understanding of the complex mechanisms involved in the determination of LR asymmetry in early development.

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Supplementary figures

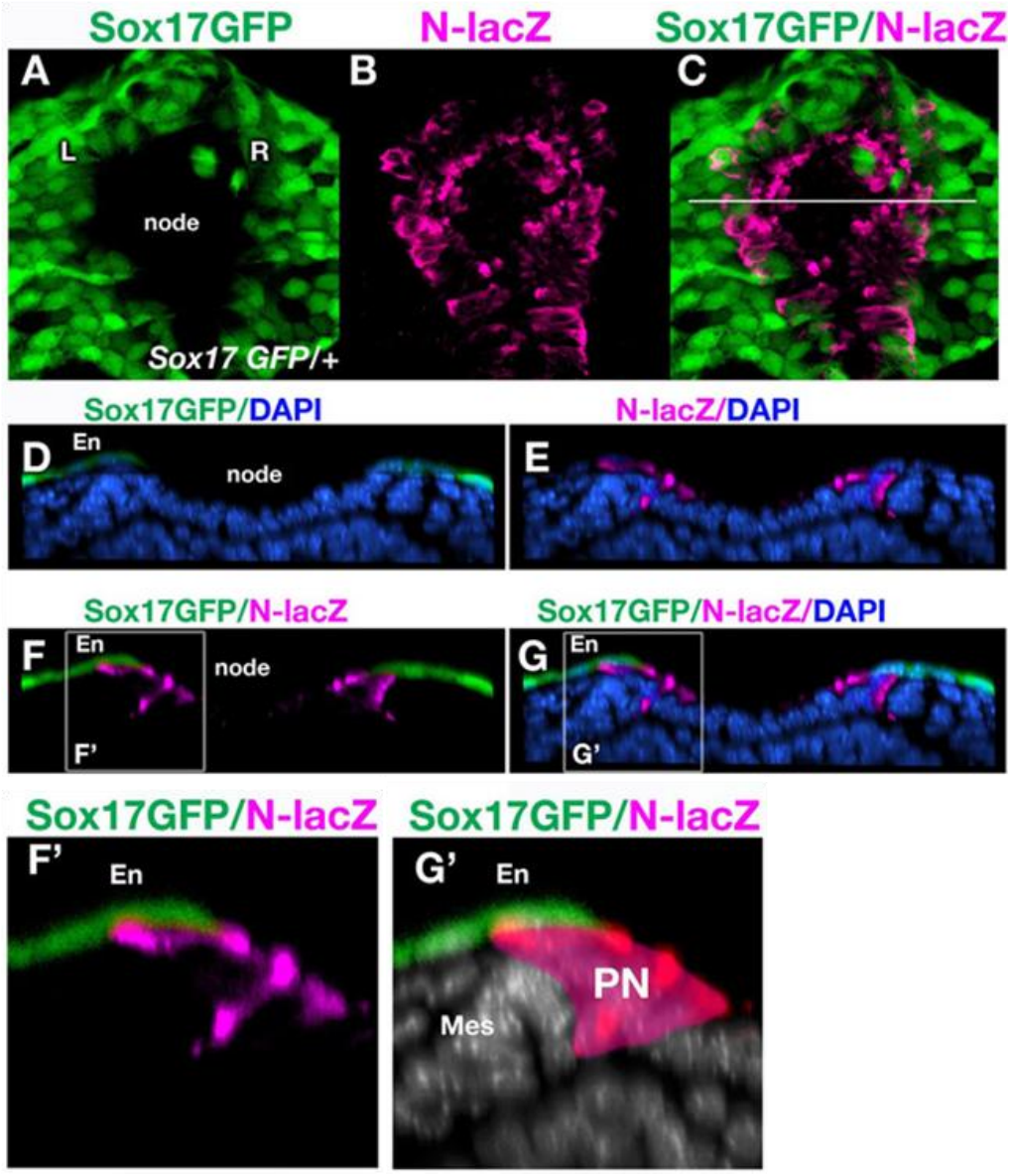

Figure S3.1 SOX17 is not expressed in perinode cells. (A-C) Whole-mount immunohistochemistry for GFP showing SOX17GFP-expressing endoderm cells (green) in the *Sox17*$^{GFP/+}$ embryo at 3-somite stage (A, C). Perinode cells labeled with the *Nodal-lacZ* transgene (N-lacZ), in which a 13 kb upstream region of the *Nodal* gene including the node enhancer is linked to a *lacZ* reporter, were visualized with anti-β-galactosidase antibody (B, C, magenta) (Adachi et al., 1999). Nuclei were stained with DAPI (blue in D, E, G). (D-G′) Optical *z*-sections at the position indicated in C (white line). (D) GFP and DAPI; (E) β-galactosidase and DAPI; (F) GFP and β-galactosidase; (G) GFP, β-galactosidase and DAPI. The perinode region is enlarged in F′ and G′ from F and G, respectively. The perinode region is shown in magenta in G′. Note that SOX17GFP expression (green) does not overlap with *Nodal-lacZ*-expressing perinode cells (magenta in F′, G′), indicating that SOX17 is not expressed in perinode cells. En, endoderm; Mes, mesoderm; PN, perinode cells.

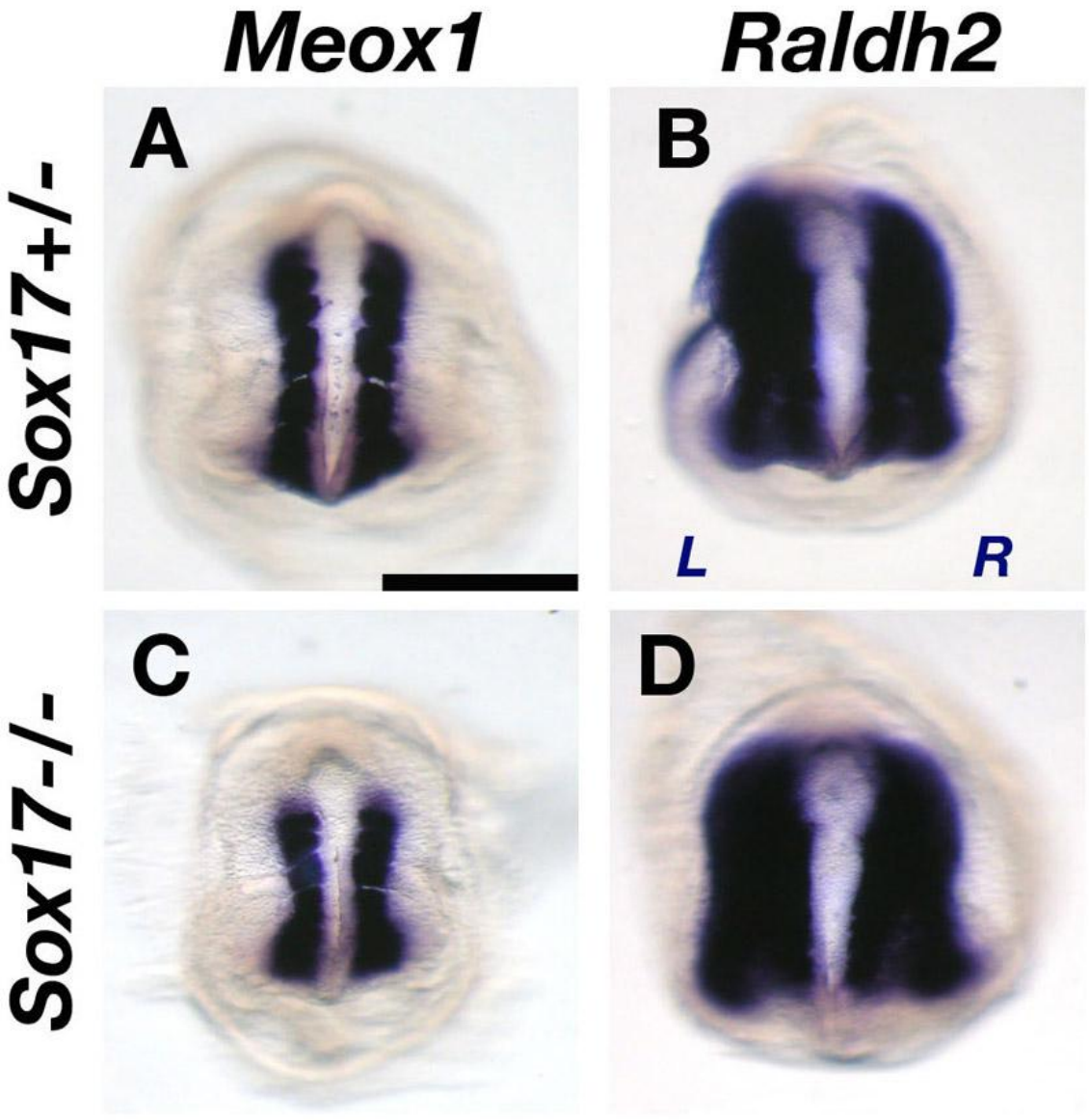

Figure S3.2 Paraxial mesoderm is properly specified in *Sox17* mutants. *Meox1* (A, C) and *Raldh2* (B, D) were expressed normally in the somite and paraxial mesoderm of $Sox17^{+/-}$ and $Sox17^{-/-}$ embryos at 5- to 6-somite stage. Scale bar: 500 μm.

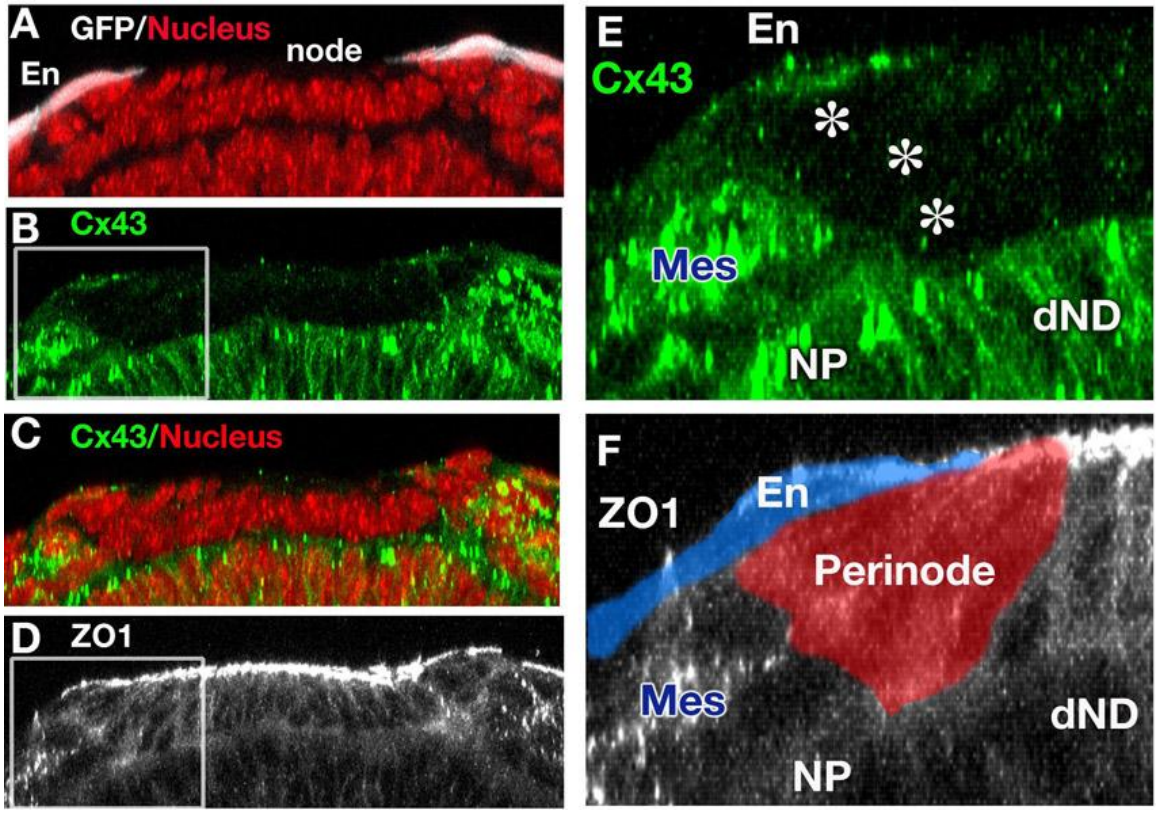

Figure S3.3 Connexin 43 (CX43) is not expressed in the perinode cells of *Sox17*$^{GFP/+}$ embryos. (A-F) Immunohistochemistry for GFP (A), CX43 (B, C, E) and ZO1 (D, F), and DAPI staining visualized endoderm (white), gap junctions (green), cell boundary (white) and nuclei (red), respectively, in *Sox17*$^{GFP/+}$ embryos. Regions enclosed by rectangles in B and D are enlarged in E and F, respectively. CX43 was expressed in endoderm (En), mesoderm (Mes), the neural plate (NP) including the dorsal layer of the node (dND), but not in the ventral node and perinode cells (stars) of *Sox17*$^{GFP/+}$ embryos (E). The positions of endoderm (GFP-positive cells, blue) and the perinode cells (red) are shown with ZO1 staining in F. The position of the perinode cells (F) was predicted based on results shown in Figure S3.1.

References

Adachi, H., Saijoh, Y., Mochida, K., Ohishi, S., Hashiguchi, H., Hirao, A. and Hamada, H. (1999). Determination of left/right asymmetric expression of nodal by a left side-specific enhancer with sequence similarity to a lefty-2 enhancer. *Genes Dev.* **13**, 1589-1600.

Baum, B. and Georgiou, M. (2011). Dynamics of adherens junctions in epithelial establishment, maintenance, and remodeling. *J. Cell Biol.* **192**, 907-917.

Beahm, D. L., Oshima, A., Gaietta, G. M., Hand, G. M., Smock, A. E., Zucker, S. N., Toloue, M. M., Chandrasekhar, A., Nicholson, B. J. and Sosinsky, G. E. (2006). Mutation of a conserved threonine in the third transmembrane helix of alpha- and beta-connexins creates a dominant-negative closed gap junction channel. *J. Biol. Chem.* **281**, 7994-8009.

Beddington, R. S. and Robertson, E. J. (1999). Axis development and early asymmetry in mammals. *Cell* **96**, 195-209.

Ben-Haim, N., Lu, C., Guzman-Ayala, M., Pescatore, L., Mesnard, D., Bischofberger, M., Naef, F., Robertson, E. J. and Constam, D. B. (2006). The nodal precursor acting via activin receptors induces mesoderm by maintaining a source of its convertases and BMP4. *Dev. Cell* **11**, 313-323.

Bre, M. H., Pepperkok, R., Hill, A. M., Levilliers, N., Ansorge, W., Stelzer, E. H. and Karsenti, E. (1990). Regulation of microtubule dynamics and nucleation during polarization in MDCK II cells. *J. Cell Biol*. **111**, 3013-3021.

Brennan, J., Norris, D. P. and Robertson, E. J. (2002). Nodal activity in the node governs left-right asymmetry. *Genes Dev*. **16**, 2339-2344.

Candia, A. F., Hu, J., Crosby, J., Lalley, P. A., Noden, D., Nadeau, J. H. and Wright, C. V. (1992). Mox-1 and Mox-2 define a novel homeobox gene subfamily and are differentially expressed during early mesodermal patterning in mouse embryos. *Development* **116**, 1123-1136.

Chen, Y. and Schier, A. F. (2001). The zebrafish Nodal signal Squint functions as a morphogen. *Nature* **411**, 607-610.

Collignon, J., Varlet, I. and Robertson, E. J. (1996). Relationship between asymmetric nodal expression and the direction of embryonic turning. *Nature* **381**, 155-158.

Constam, D. B. (2009). Running the gauntlet: an overview of the modalities of travel employed by the putative morphogen Nodal. *Curr. Opin. Genet. Dev*. **19**, 302-307.

Feistel, K. and Blum, M. (2008). Gap junctions relay FGF8-mediated right-sided repression of Nodal in rabbit. *Dev. Dyn*. **237**, 3516-3527.

Hadjantonakis, A. K., Pisano, E. and Papaioannou, V. E. (2008). Tbx6 regulates left/right patterning in mouse embryos through effects on nodal cilia and perinodal signaling. *PLoS ONE* **3**, e2511.

Hamada, H., Meno, C., Watanabe, D. and Saijoh, Y. (2002). Establishment of vertebrate left-right asymmetry. *Nat. Rev. Genet*. **3**, 103-113.

Hashimoto, H., Rebagliati, M., Ahmad, N., Muraoka, O., Kurokawa, T., Hibi, M. and Suzuki, T. (2004). The Cerberus/Dan-family protein Charon is a negative regulator of Nodal signaling during left-right patterning in zebrafish. *Development* **131**, 1741-1753.

Hou, J., Yashiro, K., Okazaki, Y., Saijoh, Y., Hayashizaki, Y. and Hamada, H. (2004). Identification of a novel left-right asymmetrically expressed gene in the mouse belonging to the BPI/PLUNC superfamily. *Dev. Dyn*. **229**, 373-379.

Kanai, Y., Kanai-Azuma, M., Noce, T., Saido, T. C., Shiroishi, T., Hayashi, Y. and Yazaki, K. (1996). Identification of two Sox17 messenger RNA isoforms, with and without the high mobility group box region, and their differential expression in mouse spermatogenesis. *J. Cell Biol.* **133**, 667-681.

Kanai-Azuma, M., Kanai, Y., Gad, J. M., Tajima, Y., Taya, C., Kurohmaru, M., Sanai, Y., Yonekawa, H., Yazaki, K., Tam, P. P. et al. (2002). Depletion of definitive gut endoderm in Sox17-null mutant mice. *Development* **129**, 2367- 2379.

Kawasumi, A., Nakamura, T., Iwai, N., Yashiro, K., Saijoh, Y., Belo, J. A., Shiratori, H. and Hamada, H. (2011). Left-right asymmetry in the level of active Nodal protein produced in the node is translated into left-right asymmetry in the lateral plate of mouse embryos. *Dev. Biol.* **353**, 321-330.

Kim, I., Saunders, T. L. and Morrison, S. J. (2007). Sox17 dependence distinguishes the transcriptional regulation of fetal from adult hematopoietic stem cells. *Cell* **130**, 470-483.

Le Good, J. A., Joubin, K., Giraldez, A. J., Ben-Haim, N., Beck, S., Chen, Y., Schier, A. F. and Constam, D. B. (2005). Nodal stability determines signaling range. *Curr. Biol.* **15**, 31-36.

Levin, M. and Mercola, M. (1998). Gap junctions are involved in the early generation of left-right asymmetry. *Dev. Biol.* **203**, 90-105.

Levin, M. and Mercola, M. (1999). Gap junction-mediated transfer of left-right patterning signals in the early chick blastoderm is upstream of Shh asymmetry in the node. *Development* **126**, 4703-4714.

Mahlapuu, M., Ormestad, M., Enerback, S. and Carlsson, P. (2001). The forkhead transcription factor Foxf1 is required for differentiation of extraembryonic and lateral plate mesoderm. *Development* **128**, 155-166.

Marjoram, L. and Wright, C. (2011). Rapid differential transport of Nodal and Lefty on sulfated proteoglycan-rich extracellular matrix regulates left-right asymmetry in Xenopus. *Development* **138**, 475-485.

McGrath, J., Somlo, S., Makova, S., Tian, X. and Brueckner, M. (2003). Two populations of node monocilia initiate left-right asymmetry in the mouse. *Cell* **114**, 61-73.

Meno, C., Saijoh, Y., Fujii, H., Ikeda, M., Yokoyama, T., Yokoyama, M., Toyoda, Y. and Hamada, H. (1996). Left-right asymmetric expression of the TGF betafamily member lefty in mouse embryos. *Nature* **381**, 151-155.

Mercola, M. (2003). Left-right asymmetry: nodal points. *J. Cell Sci.* **116**, 3251-3257.

Nakamura, T., Mine, N., Nakaguchi, E., Mochizuki, A., Yamamoto, M., Yashiro, K., Meno, C. and Hamada, H. (2006). Generation of robust left-right asymmetry in the mouse embryo requires a self-enhancement and lateral-inhibition system. *Dev. Cell* **11**, 495-504.

Niederreither, K., McCaffery, P., Drager, U. C., Chambon, P. and Dolle, P. (1997). Restricted expression and retinoic acid-induced downregulation of the retinaldehyde dehydrogenase type 2 (RALDH-2) gene during mouse development. *Mech. Dev*. **62**, 67-78.

Nonaka, S., Tanaka, Y., Okada, Y., Takeda, S., Harada, A., Kanai, Y., Kido, M. and Hirokawa, N. (1998). Randomization of left-right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. *Cell* **95**, 829-837.

Oki, S., Hashimoto, R., Okui, Y., Shen, M. M., Mekada, E., Otani, H., Saijoh, Y. and Hamada, H. (2007). Sulfated glycosaminoglycans are necessary for Nodal signal transmission from the node to the left lateral plate in the mouse embryo. *Development* **134**, 3893-3904.

Osada, S. I., Saijoh, Y., Frisch, A., Yeo, C. Y., Adachi, H., Watanabe, M., Whitman, M., Hamada, H. and Wright, C. V. (2000). Activin/nodal responsiveness and asymmetric expression of a Xenopus nodal-related gene converge on a FAST-regulated module in intron 1. *Development* **127**, 2503-2514.

Pearce, J. J., Penny, G. and Rossant, J. (1999). A mouse Cerberus/Dan-related gene family. *Dev. Biol*. **209**, 98-110.

Pfister, S., Jones, V. J., Power, M., Truisi, G. L., Khoo, P. L., Steiner, K. A., KanaiAzuma, M., Kanai, Y., Tam, P. P. and Loebel, D. A. (2011). Sox17-dependent gene expression and early heart and gut development in Sox17-deficient mouse embryos. *Int. J. Dev. Biol*. **55**, 45-58.

Rankin, C. T., Bunton, T., Lawler, A. M. and Lee, S. J. (2000). Regulation of leftright patterning in mice by growth/differentiation factor-1. *Nat. Genet*. **24**, 262-265.

Saijoh, Y., Adachi, H., Sakuma, R., Yeo, C. Y., Yashiro, K., Watanabe, M., Hashiguchi, H., Mochida, K., Ohishi, S., Kawabata, M. et al. (2000). Left-right asymmetric expression of lefty2 and nodal is induced by a signaling pathway that includes the transcription factor FAST2. *Mol. Cell* **5**, 35-47.

Saijoh, Y., Oki, S., Ohishi, S. and Hamada, H. (2003). Left-right patterning of the mouse lateral plate requires nodal produced in the node. *Dev. Biol*. **256**, 160-172.

Saund, R. S., Kanai-Azuma, M., Kanai, Y., Kim, I., Lucero, M. T. and Saijoh, Y. (2012). Gut endoderm is involved in the transfer of left-right asymmetry from the node to the lateral plate mesoderm in the mouse embryo. *Development* **139**(13): 2426-35.

Sakamoto, Y., Hara, K., Kanai-Azuma, M., Matsui, T., Miura, Y., Tsunekawa, N., Kurohmaru, M., Saijoh, Y., Koopman, P. and Kanai, Y. (2007). Redundant roles of Sox17 and Sox18 in early cardiovascular development of mouse embryos. *Biochem. Biophys. Res. Commun.* **360**, 539-544.

Shaw, R. M., Fay, A. J., Puthenveedu, M. A., von Zastrow, M., Jan, Y. N. and Jan, L. Y. (2007). Microtubule plus-end-tracking proteins target gap junctions directly from the cell interior to adherens junctions. *Cell* **128**, 547-560.

Shen, M. M., Wang, H. and Leder, P. (1997). A differential display strategy identifies Cryptic, a novel EGF-related gene expressed in the axial and lateral mesoderm during mouse gastrulation. *Development* **124**, 429-442.

Sulik, K., Dehart, D. B., Iangaki, T., Carson, J. L., Vrablic, T., Gesteland, K. and Schoenwolf, G. C. (1994). Morphogenesis of the murine node and notochordal plate. *Dev. Dyn*. **201**, 260-278.

Tanaka, C., Sakuma, R., Nakamura, T., Hamada, H. and Saijoh, Y. (2007). Long-range action of Nodal requires interaction with GDF1. *Genes Dev*. **21**, 3272-3282.

Tanaka, Y., Okada, Y. and Hirokawa, N. (2005). FGF-induced vesicular release of Sonic Hedgehog and retinoic acid in leftward nodal flow is critical for left-right determination. *Nature* **435**, 172-177.

Thomas, T., Jordan, K., Simek, J., Shao, Q., Jedeszko, C., Walton, P. and Laird, D. W. (2005). Mechanisms of Cx43 and Cx26 transport to the plasma membrane and gap junction regeneration. *J. Cell Sci*. **118**, 4451-4462.

Tremblay, K. D., Hoodless, P. A., Bikoff, E. K. and Robertson, E. J. (2000). Formation of the definitive endoderm in mouse is a Smad2-dependent process. *Development* **127**, 3079-3090.

Tsiairis, C. D. and McMahon, A. P. (2009). An Hh-dependent pathway in lateral plate mesoderm enables the generation of left/right asymmetry. *Curr. Biol*. **19**, 1912-1917.

Vermot, J. and Pourquie, O. (2005). Retinoic acid coordinates somitogenesis and left-right patterning in vertebrate embryos. *Nature* **435**, 215-220.

Viotti, M., Niu, L., Shi, S. H. and Hadjantonakis, A. K. (2012). Role of the gut endoderm in relaying left-right patterning in mice. *PLoS Biol*. **10**, e1001276.

Wilkinson, D. G. (1998). In situ hybridization – a practical approach. Oxford: Oxford University Press. New York, USA.

Yan, Y. T., Gritsman, K., Ding, J., Burdine, R. D., Corrales, J. D., Price, S. M., Talbot, W. S., Schier, A. F. and Shen, M. M. (1999). Conserved requirement for EGF-CFC genes in vertebrate left-right axis formation. *Genes Dev*. **13**, 2527-2537.

Yoshioka, H., Meno, C., Koshiba, K., Sugihara, M., Itoh, H., Ishimaru, Y., Inoue, T., Ohuchi, H., Semina, E. V., Murray, J. C. et al. (1998). Pitx2, a bicoidtype homeobox gene, is involved in a lefty-signaling pathway in determination of left-right asymmetry. *Cell* **94,** 299-305.

Yost HJ. (1990). Inhibition of proteoglycan synthesis eliminates left-right asymmetry in Xenopus laevis cardiac looping. *Development*. **110**(3):865-74.

CHAPTER 4

SOX17 FUNCTIONS AS A NEGATIVE REGULATOR OF *NODAL* IN THE DEFINITIVE ENDODERM AT THE PRIMITIVE STREAK STAGES

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Abstract

Definitive endoderm arises from ingression of epiblast cells at the primitive streak and expresses *Nodal*, which is then rapidly suppressed in the definitive endoderm cells as they begin to differentiate. SOX17, an HMG-box transcription factor, begins expression in the definitive endoderm at the streak stage and expands to become localized in the foregut at early somite stages. We found that in *Sox17* mutants, *Nodal* expression persists in the endoderm cells at poststreak stages and becomes ectopically localized in the foregut at the early somite stages, suggesting SOX17 is involved in the suppression of *Nodal* in the definitive endoderm in the normal embryo. Consequently, we have investigated the regulatory mechanisms that control the ectopic expression of *Nodal* in *Sox17* mutants. We found that a novel endoderm-specific enhancer regulates *Nodal* expression in the definitive endoderm at the streak stages. This enhancer is rapidly

suppressed by the late bud stages in normal embryos, but remains active in *Sox17* mutants until the early somite stages. Significantly, Nodal signaling was also found to be activated in the foregut endoderm in *Sox17* mutants at early somite stages, potentially amplifying *Nodal* expression in the mutant foregut endoderm. In this study, we report that SOX17 functions as a negative regulator of *Nodal* in the definitive endoderm at streak stages, and a two-step regulatory mechanism is responsible for the ectopic *Nodal* expression in the *Sox17* mutant endoderm.

Introduction

The progenitor cells that arise during gastrulation proliferate and differentiate to form complex organ systems that ultimately shape the entire embryo. Definitive endoderm, one of the three germ layers, gives rise to specialized epithelial cell types that line the digestive tract such as the stomach and the intestines. Gastrulation in mouse embryos begins when the primitive streak appears at the posterior side of the embryo at E6.5, where ingressing epiblast cells undergo an epithelial-mesenchymal transition (EMT) to form the mesoderm and the definitive endoderm. Then the definitive endoderm cells intercalate into the overlying visceral endoderm and populate the entire embryonic region, dispersing the visceral endoderm amongst the definitive endoderm cells (Arnold and Robertson, 2009; Kwon et al., 2008). It is now well established that NODAL signaling is required for the specification of endoderm as well as mesoderm at the primitive streak (Tremblay et al., 2000). Higher NODAL activity has been shown to be important for definitive endoderm differentiation, whereas lower levels of NODAL activity promotes mesoderm differentiation (Costello et al., 2011; Gadue et al., 2006)

The A-P axis in the mouse embryos is established at E6.5, with the anterior visceral endoderm (AVE) expressing NODAL antagonists marking the anterior side and the epiblast located at the opposite end forms the primitive streak (Perea-Gomez et al., 2002). *Nodal* is expressed in the primitive streak and *Nodal* null mutants fail to form a primitive streak (Conlon et al., 1994). As the primitive streak elongates, *Nodal* is robustly expressed along the entire length of the primitive streak through activation of the Nodal signaling pathway, which is mediated by SMAD2/FOXH1 transcription factor complex by binding the asymmetric enhancer element (ASE) of *Nodal* (Norris and Robertson, 1999; Norris et al., 2002; Saijoh et al., 2000). Conditional deletion of *Smad2* in the epiblast leads to specific loss of the definitive endoderm cells but does not affect primitive streak formation, suggesting the central requirement of Nodal signaling for the patterning of definitive endoderm in the primitive streak (Norris et al., 2002; Vincent et al., 2003). Definitive endoderm develops from mesendoderm precursors that express *Nodal* at the primitive streak margin, and the emergent endoderm can be visualized by the expression of endoderm regulator genes, *Foxa2* and *Sox17*. Both these genes are expressed in the definitive endoderm; however, *Foxa2* is also expressed in the anterior primitive streak and midline structures (Hallonet et al., 2002; Saund et al., 2012). *Sox17* is the only known gene that is specifically expressed in endoderm cells after they migrate from the primitive streak. At the late bud stage (E7.5), *Sox17* is expressed in the entire definitive endoderm except the distal region and overlying the primitive streak (Saund et al., 2012). Although the upstream regulators of *Sox17* in the mouse embryo have not yet been characterized, studies from Xenopus and zebrafish reveal that *Sox17* expression in the endoderm is regulated by the Nodal signaling pathway (Woodland and Zorn, 2008).

Our studies show that *Nodal* was found to be expressed in the newly originated definitive endoderm cells that intercalate into the outer visceral endoderm layer, in the posterior embryonic region in close proximity to the primitive streak (unpublished observations). However, the expression is repressed as the cells move anteriorly between the bud and headfold stages. These endoderm cells begin to form the characteristic epithelial morphology of the definitive endoderm and express endoderm-specific genes such as *Sox17*, coincident with the loss of *Nodal* expression at the early bud stages.

In the present study, we found that *Nodal* expression in endoderm continued from the primitive streak to the early somite stages in *Sox17* mutants, suggesting that SOX17 is involved in the suppression of *Nodal* expression in the endoderm cells. We have examined how *Nodal* expression is regulated in the endoderm in normal embryos and found a novel endoderm-specific cis-regulatory element in the *Nodal* genomic locus responsible for the *Nodal* expression at the streak stages, which may be negatively regulated by SOX17. The conditional deletion of *Sox17* in the entire embryo at the poststreak stages provided evidence for a direct role of SOX17 in the suppression of *Nodal* in the definitive endoderm. Our studies also show activation of the Nodal signaling pathway via positive and negative feedback loops at the early somite stages in the mutant endoderm, revealing a two-step stage-specific regulatory process that results in the ectopic expression of *Nodal* in the foregut in *Sox17* mutants. These observations indicate a fundamental requirement for the downregulation of *Nodal* in the definitive endoderm at the early stages, regulated by the downstream endoderm regulator SOX17, which may be necessary for subsequent endoderm differentiation at later stages of development.

Nodal ectopic expression in foregut endoderm originates at the gastrulation stage in *Sox17* mutant embryos

Sox17 is an essential gene for the formation and differentiation of definitive endoderm in developing mouse embryos (Kanai-Azuma et al., 2002). SOX17 functions to maintain the epithelial shape and cell-cell communication necessary for proper differentiation of definitive endoderm cells (Saund et al., 2012). In this study, we showed that defects in endoderm morphology in *Sox17* mutants lead to loss of the relay of a LR signal, necessary for the asymmetric expression of *Nodal* in the LPM.

Here, we show that *Nodal* expression was ectopically activated in scattered foregut endoderm cells in *Sox17* mutant embryos at E8.5 (Figure 4.1E-F'). In addition, the direct downstream targets of NODAL, *Lefty2* and *Pitx2,* were also expressed in the foregut endoderm. *Pitx2* was expressed broadly in the foregut pocket, whereas *Lefty2* showed a patchy expression pattern encompassing the entire foregut endoderm (Saund et al., 2012). During gastrulation, *Nodal* is expressed in the primitive streak and is required for the specification of definitive endoderm. We then examined the temporal activation of ectopic *Nodal* expression in endoderm at different stages post gastrulation to determine whether the ectopic expression appears at the foregut at E8.5 or if it continues from the gastrulation stages. We found that at the late bud stage (E7.5), *Nodal* expression domain was broader and stronger in the endoderm cells in *Sox17* mutants, compared to normal embryos in which *Nodal* expression was turned off in endoderm cells (Figure 4.1A-B'), suggesting that the *Nodal* expression in the endoderm cells continued from the primitive streak stage during early gastrulation. At the 1- to 2-somite stages (E8.25), the definitive endoderm cells colonized the foregut, where *Nodal* was still found to be strongly

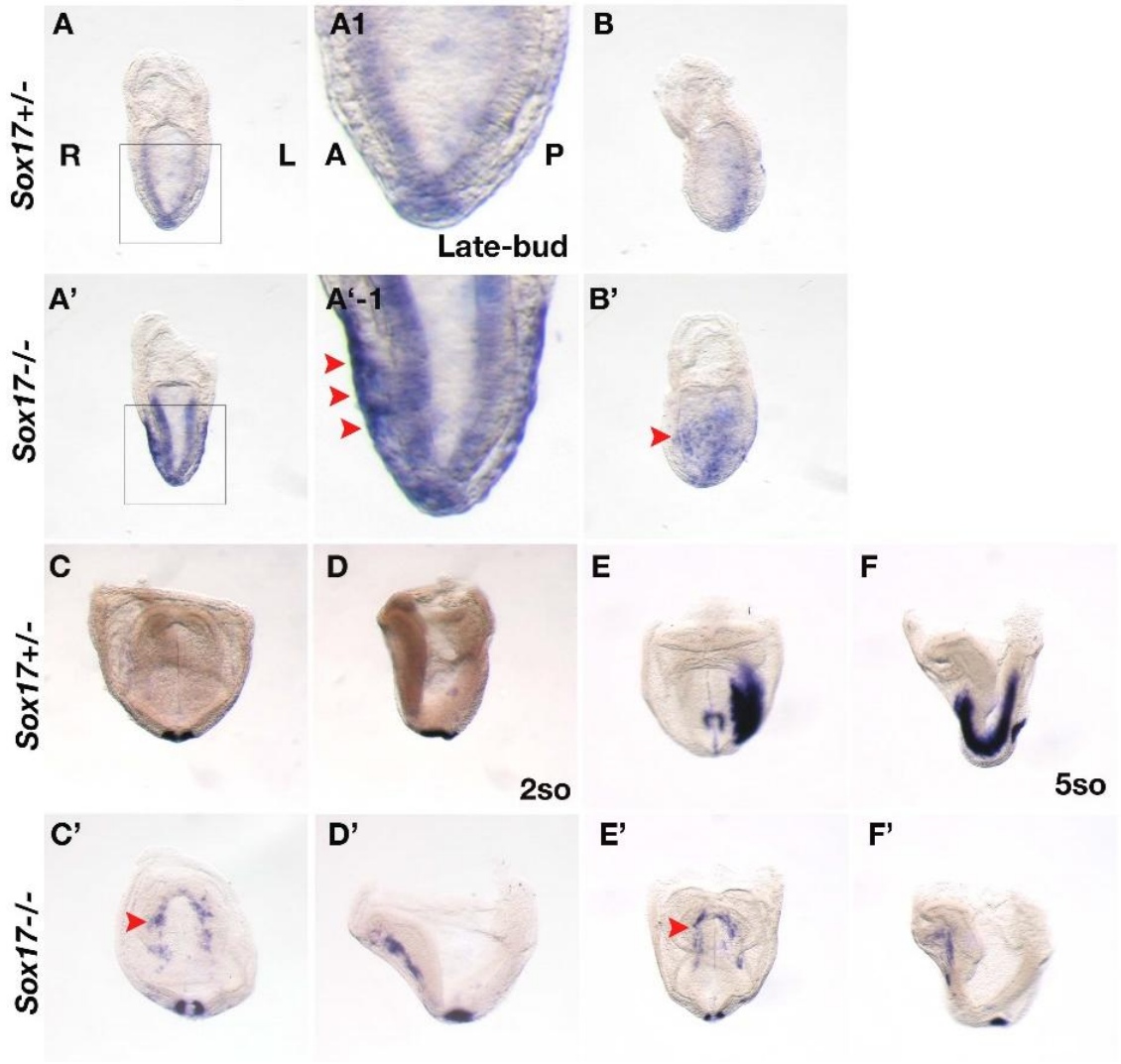

Figure 4.1 *Nodal* is ectopically expressed in *Sox17* mutants from late bud to somite stages (5-somite stage). (A, B) Anterior and left-side views of a late bud embryo (E7.5) showing *Nodal* expression in the primitive streak, compared to a *Sox17* mutant embryo (A', B'; magnified insets A-1, A'-1) at the late bud stage showing *Nodal* expression in the primitive streak and higher levels in discrete cells in the endoderm layer (red arrowheads in A'-1. (C-F') Anterior and left-side view of a normal 2-somite stage (C, D) and 5-somite stage embryo with *Nodal* expression in the node (C, D) and in left LPM (E, F), and *Sox17* mutants at 2-somite stage (C', D', red arrowhead) and 5-somite stage (E', F', red arrowhead) showing strong *Nodal* expression in the foregut endoderm, apart from the node.

expressed in the *Sox17* mutants (Figure 4.1C-D'). Thus, the question remains, how does SOX17 regulate the expression of *Nodal* in the definitive endoderm cells? To address this question, we then examined the transcriptional regulation of *Nodal* gene in *Sox17* mutants.

Nodal signaling is responsible for ectopic expression of *Nodal* in the foregut during somite stages.

The foregut in *Sox17* mutants showed ectopic expression of not only *Nodal* but also the direct downstream targets, *Lefty2* and *Pitx2,* at E8.5 (Chapter 3, Figure 3.1). These observations suggested the presence of an active NODAL feedback signaling pathway in the foregut, similar to that in the LPM in normal embryos, established by the positive and negative feedback mechanisms of Nodal signaling involving *Lefty2* and *Pitx2* (Hamada et al., 2002). This transcriptional regulation depends on the asymmetric enhancer (ASE) element containing FOXH1 binding sites that are present in *Nodal, Lefty2 and Pitx2* genes (Adachi et al., 1999; Saijoh et al., 2000; Shiratori et al., 2001). To test whether the Nodal signaling is active in the foregut of *Sox17* mutants, we introduced the *Lefty2-ASE* transgene into *Sox17* heterozygous mice. This *Lefty2-ASE* enhancer has been well characterized and is thought to be a NODAL signal responsive element through FOXH1 (Saijoh et al., 2000).

In normal embryos, *Lefty2-ASE* is strongly expressed in the primitive streak at the late streak stage (E7.0) and recapitulates *Nodal* expression, but it is absent at the late bud stage (E7.5) and the headfold stages (E8.0) (Figure 4.2A; Saijoh et al., 2000). At the 5- to 6-somite stages, this transgene was active in the left LPM, similar to *Nodal* and *Lefty2* expression (Figure 4.2B). In *Sox17* mutants, the *Lefty2-ASE* was strongly expressed in the

foregut endoderm at 5-somite stage (Figure 4.2B'), but absent in the left LPM, clearly establishing that Nodal signaling is active in the foregut in the *Sox17* mutant embryos. Hence, the ASE element is responsible for the ectopic expression of *Nodal* in the *Sox17* mutant foregut endoderm at this stage. These data suggested that in normal embryos, the ASE element would be repressed by SOX17 to turn off *Nodal* expression in the endoderm. Since *Nodal* was ectopically expressed at the late bud stage in *Sox17* mutants, we then examined the *Lefty2-ASE* transgene at an earlier stage of endoderm differentiation in the *Sox17* mutants. Surprisingly, we found that the *Lefty2-ASE* transgene was completely absent in the mutant embryos at the headfold stage (E7.5) (Figure 4.2A'), indicating that the ASE is not involved in the initiation of ectopic *Nodal* expression; however, it is responsible for the maintenance of higher levels of ectopic *Nodal* expression at the early somite stages in the *Sox17* mutants. Thus, it is likely that regulation of the ectopic *Nodal* expression in the endoderm in *Sox17* mutants is initiated by a separate mechanism (Figure 4.1A).

Nodal expression in endoderm is initiated by a novel endoderm-specific cis-regulatory element in *Sox17* mutants

To address how *Nodal* ectopic expression is triggered in *Sox17* mutants, we searched for possible candidate enhancer elements other than ASE in the *Nodal* genomic region. The 13kb region upstream of *Nodal* transcriptional start site contains the known node specific enhancer element (NDE), the left-side LPM enhancer (LSE) and the proximal epiblast enhancer (PEE) (Adachi et al., 1999; Saijoh et al., 2003; Vincent et al., 2003). A reporter transgene containing the 13 kb fragment linked to *LacZ* (*Nodal*-5'-*LacZ*) was introduced into *Sox17* heterozygous mutant mice. In control embryos at E6.5,

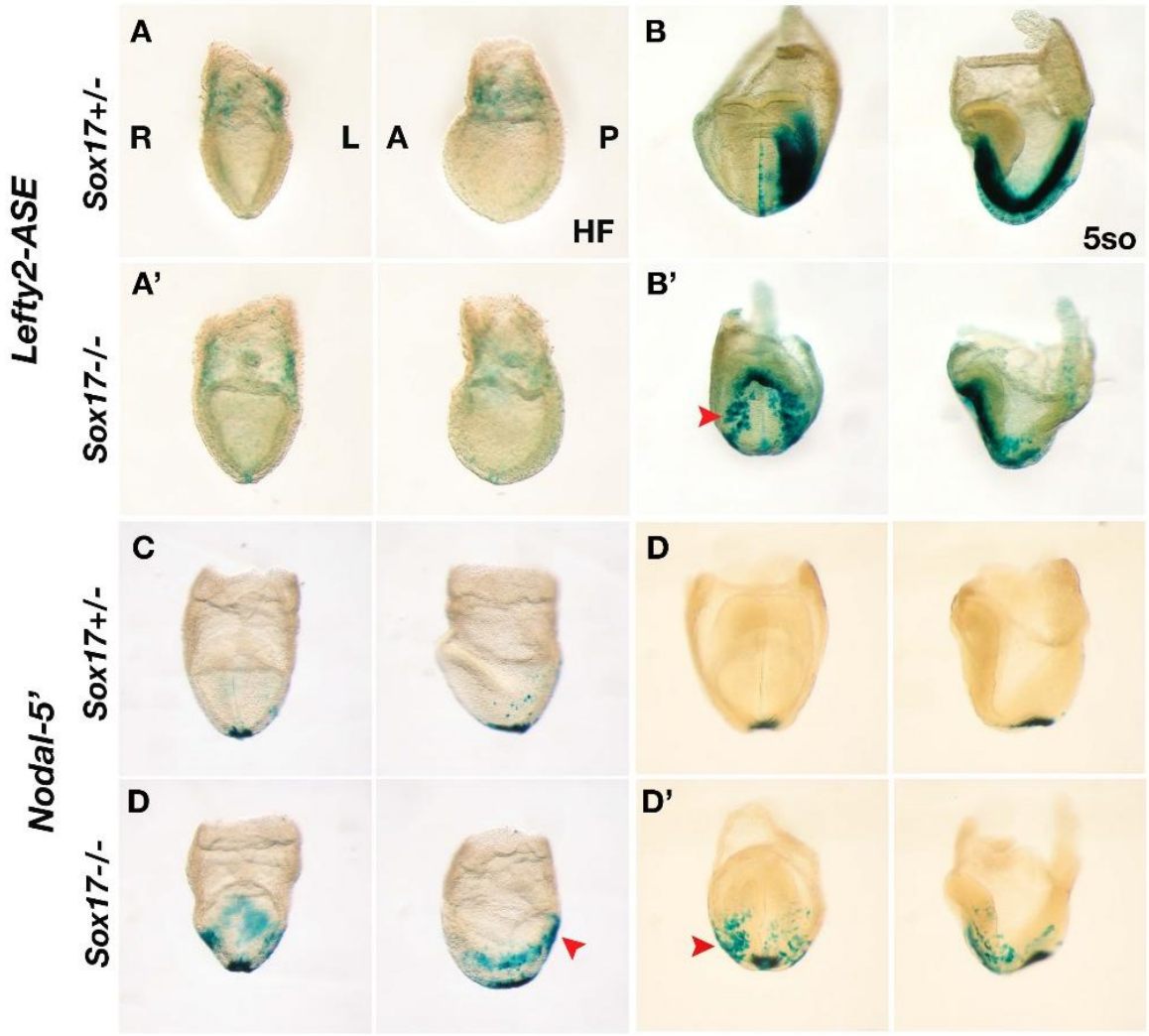

Figure 4.2 Ectopic *Nodal* expression is driven by Nodal signaling at the 5-somite stage (E8.5) and by a novel regulatory element at the headfold stages (E8.0) in *Sox17* mutants. The *Lefty2-ASE* transgenic reporter for Nodal signaling is absent in both normal and *Sox17* mutants (A, A') at headfold stage (HF), it is expressed in the left LPM at 5-somite stage in the normal embryo (B), and in the foregut endoderm in the *Sox17* mutant (B', red arrowhead). The *Nodal-5' LacZ* reporter transgene containing 13kb upstream fragment of *Nodal* is active only in the node in normal embryos at headfold (C) and 4-somite stage (D), whereas the transgene is also strongly expressed in the endoderm cells in *Sox17* mutant embryos (C', D', red arrowhead).

we found that this transgene was expressed in the early definitive endoderm cells that are beginning to emigrate from the primitive streak (data not shown), suggesting the presence of a cis-regulatory element in the 13 kb fragment that specifically controls the endoderm expression of *Nodal*. This is a novel and important finding that reveals possible mechanisms involving *Nodal* in the segregation of the endoderm and mesoderm lineage from precursors in the primitive streak. It has been known that endoderm differentiation in the primitive streak requires a higher NODAL activity but the regulatory mechanisms controlling *Nodal* expression are unknown (Gadue et al., 2006). Our observation suggests that the upregulated NODAL activity in endoderm may be caused by increased transcriptional activity of *Nodal* due to the endoderm specific enhancer. The transgene is not expressed in the definitive endoderm at the headfold stage (E7.5) in normal development (Figure 4.2C). This suggests that the endoderm-specific expression of *Nodal-5'-LacZ* is limited to the earliest stages of endoderm differentiation, corresponding with the endogenous *Nodal* expression at the primitive streak stage. In *Sox17* mutant embryos, the *Nodal-5'-LacZ* transgene is strongly expressed in the endoderm cells in the headfold stage, when the *Lefty2-ASE* transgene is not active (Figure 4.2C'). These data indicate that the ectopic *Nodal* expression is initiated in the endoderm through the regulatory elements found upstream of the *Nodal* gene, and Nodal signaling is not involved in ectopic *Nodal* expression at the early stage of endoderm differentiation (E7.5).

At E8.5 (3- to 4-somite stage.) the *Nodal-5'-LacZ* transgene continues to be expressed in the foregut endoderm and node in *Sox17* mutants, whereas it is restricted to the node in normal embryos (Figure 4.2D, D'). At this stage, however, Nodal signaling

becomes activated in the foregut in mutant embryos, as evidenced by the strong expression of *Lefty2-ASE* (Figure 4.2B, B'), which amplifies expression of *Nodal*, *Lefty2* and *Pitx2* in the foregut endoderm. Thus, the initial stages of ectopic expression of *Nodal* in *Sox17* mutants results from the endoderm-specific enhancer of *Nodal* found in *Nodal-5'-LacZ*, whereas at later stages (E8.5), the ectopic expression is maintained and enhanced by the activation of the ASE in the foregut endoderm. These data suggest that SOX17 is a negative regulator of *Nodal* expression in differentiating endoderm cells by repressing the endoderm enhancer of *Nodal* directly or indirectly, and they provide detailed insights into the molecular mechanisms that result in the ectopic expression of *Nodal* in the foregut endoderm in *Sox17* mutants. These studies also shed light into how *Nodal* expression is regulated in normal embryos during the early stages and then repressed in the process of definitive endoderm differentiation.

Sox17 function is required in the early definitive endoderm to prevent activation of ectopic *Nodal* expression

Sox17 is dynamically expressed in the definitive endoderm, from the primitive streak to the headfold stage, fated to form the foregut at E8.5 (Saund et al., 2012). We asked, when is SOX17 required for suppression of *Nodal* expression in endoderm cells arising from the primitive streak? In normal embryos, *Nodal* is rapidly suppressed in the definitive endoderm at E7.0, when the cells begin to migrate to the anterior region; hence, it was most likely that SOX17 functions between E6.5 to E7.0 to turn off *Nodal* expression. To test this possibility, we aimed to conditionally delete the *Sox17* gene, after *Sox17* begins to be expressed in the newly generated definitive endoderm cells from the primitive streak at E6.5. We employed the tamoxifen induced CRE mediated

recombination system driven by the endogenous *Rosa26* promoter/enhancer (*Rosa-CreER*) for the temporal control of conditional deletion of *Sox17*. The *Rosa26* promoter is expressed in all tissues, including definitive endoderm; hence, tamoxifen induced activation of *Rosa-CreER* will allow the temporal control of *Sox17* loss of function by recombination of the floxed-conditional *Sox17* mutant embryos. Previous studies had shown that tamoxifen induced deletion of *Rosa-CreER* requires approximately 12 hours for complete recombination (Park et al., 2008). We determined the efficacy of tamoxifen induced *Cre* recombination by crossing homozygous *Rosa-CreER* mice with homozygous *dTdG* Cre reporter females and tested *Cre* recombination after 12 hours of tamoxifen administration to pregnant females at E6.5, by observing the change in membrane fluorescence from DSRED to EGFP in embryos (Muzumdar et al., 2007). *Sox17*$^{GFP/+}$*;Rosa*$^{CreER/CreER}$*;Nodal* 5'*-LacZ* males were crossed to *Sox17-flox* mice, *Sox17*$^{F/F}$*;dTdG/dTdG* to obtain conditional *Sox17*$^{GFP/F}$*;Rosa*$^{CreER/+}$*;Nodal-5'-LacZ* embryos. CRE induction by oral administration of tamoxifen (10 mg/ml) to pregnant females was done at E6.5, when the embryos are at the late primitive streak stage. The embryos were harvested at the 5- to 6-somite stage (E8.5), approximately 36 hours after tamoxifen administration. In this procedure, *Sox17* is initially expressed in the definitive endoderm but is conditionally deleted at the late streak to bud stages in the migrating definitive endoderm cells. The conditional *Sox17* mutant embryos showed foregut defects that appeared similar although less severe than *Sox17* null mutant embryos (data not shown). The control and conditional mutant embryos were treated with X-gal staining to examine the expression of the *Nodal-5'-LacZ* transgene. We found that the activity of *Nodal-5'-LacZ* was vastly reduced and weak expression remained in the foregut

endoderm in *Sox17* conditional mutants at 6-somite stage, in contrast to the *Sox17* mutant embryos in which *Nodal-5'-LacZ* was strongly expressed (Figure 4.3A-B'). The significant reduction of *Nodal-5'-LacZ* activity suggests that initially expressed SOX17 at the streak stage before conditional deletion of *Sox17* prevents ectopic *Nodal* activation in the foregut endoderm to a considerable extent. Thus, SOX17 function is required in the definitive endoderm arising at the late streak stage (E6.5-7.0) for the suppression of *Nodal* expression in the definitive endoderm.

Discussion

In this study, we have focused on the regulation of Nodal signaling in the definitive endoderm in normal embryos. We have found that after definitive endoderm emerges from the primitive streak, *Nodal* expression persists over a prolonged period in endoderm in *Sox17* mutant embryos; whereas in normal embryos, *Nodal* is rapidly suppressed in definitive endoderm. The detailed expression analysis from the primitive streak to the early somite stages reveals that *Nodal* is continuously expressed in endoderm in the *Sox17* mutant embryos. To examine the regulatory mechanisms of the *Nodal* ectopic expression, we have used transgenic reporter lines to investigate the activation of Nodal signaling and endoderm-specific expression of *Nodal* in *Sox17* mutant embryos. Our results show that the ectopic *Nodal* expression at the streak to late bud stages (E7.5) is regulated by the *Nodal* endoderm specific enhancer, then at 2- to 6-somite stages, the expression is amplified by Nodal signaling via the ASE enhancer through a positive feedback mechanism resulting in amplification of *Nodal* expression and induction of *Lefty2* and *Pitx2* expression. This is a significant finding that suggests

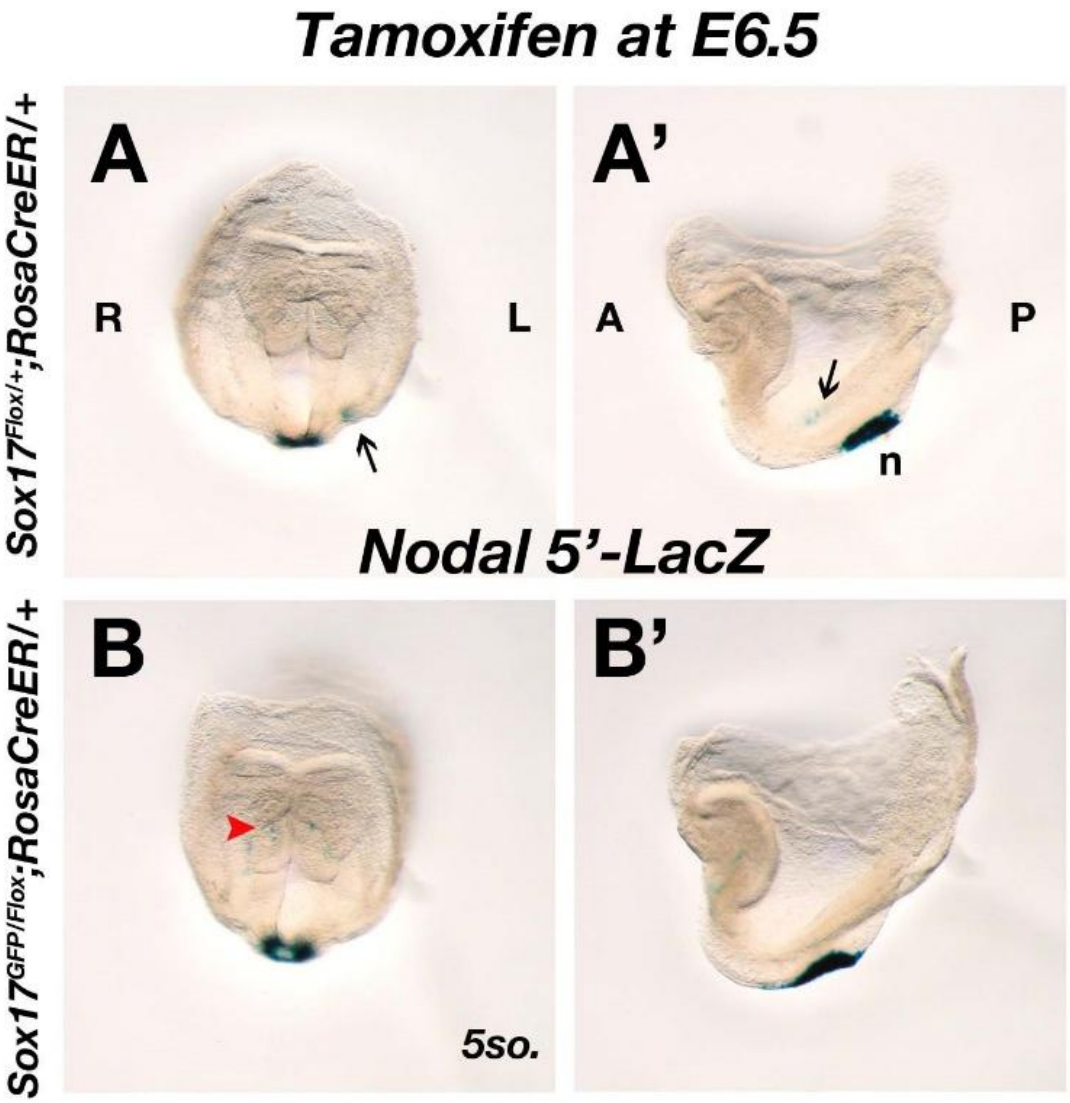

Figure 4.3 *Nodal5'-LacZ* is not expressed in the foregut endoderm, when *Sox17* is conditionally deleted at E6.5 in the entire embryo. *Sox17*$^{Flox/+}$*;RosaCreER/+ and* *Sox17*$^{GFP/Flox}$*;RosaCreER/+* embryos obtained from cross of *Sox17*$^{GFP/+}$*;RosaCre*$^{ER/ER}$*;Nodal 5'-LacZ* with *Sox17*$^{Flox/Flox}$*;dTdG/dTdG*, and administered with tamoxifen to pregnant mother at E6.5 and harvested at 5-somite stage and treated with X-gal staining. The ectopic expression of *Nodal* 5'*-LacZ* in the foregut was absent in control (A, A') and very weak in conditional mutant embryos (B, B', red arrowhead). The arrows in A, A' indicate weak expression of *Nodal* 5'*-LacZ* in the left LPM. n=node.

SOX17 is a negative regulator of *Nodal* in definitive endoderm differentiation and reveals the presence of a novel endoderm-specific regulatory mechanism for *Nodal* expression.

SOX17 may directly repress *Nodal* expression in the early definitive endoderm

The ectopic expression of *Nodal* in *Sox17* mutants from the primitive streak to the early somite stages raises the possibility that SOX17 may directly or indirectly regulate *Nodal* expression. We have examined the upstream region of *Nodal* using transgenic reporters in *Sox17* mutants and found that the entire 13 kb fragment (*Nodal 5'-LacZ*) showed robust ectopic expression, suggesting a novel regulatory element directs *Nodal* expression in the definitive endoderm. The Proximal Epiblast enhancer (*PEE*, 2 kb) element of *Nodal* has been shown to initiate *Nodal* expression at the proximal epiblast and the initial primitive streak at E5.5 under the control of WNT3 via TCF binding sites (Ben-Haim et al., 2006). The *Nodal 5'-LacZ* reporter showing ectopic expression in *Sox17* mutants contains the *PEE* element; we predict that the definitive endoderm-specific *Nodal* expression is driven by regulatory elements in the *PEE*, although cis-analysis of the entire 13kb *Nodal 5'-LacZ* fragment will be required to reveal the minimal element responsible for endoderm expression.

We still do not know whether the regulation of *Nodal* expression by SOX17 is direct or indirect. We have found several potential SOX17 binding sites upstream of *Nodal*, including in the *Nodal* PEE element, in close conjunction with the TCF sites, that suggest the possible mechanisms by which SOX17 may directly suppress *Nodal* expression in definitive endoderm. We propose the following three models which may explain how SOX17 can directly repress Wnt-signaling mediated *Nodal* expression: 1)

SOX17 may bind and sequester beta-catenin to prevent the transcriptional activation by interaction of beta-catenin with TCF factors; 2) SOX17 may directly compete with TCF factors for binding to the HMG-box DNA binding sequences in PEE; 3) SOX17 binds to the SOX-specific binding sites that are in close proximity to the TCF binding sites in the *Nodal* genomic region, forming heterodimers with TCF factors, which may interfere with beta-catenin mediated transcriptional activation of target genes (Liu et al., 2007; Liu et al., 2010; Sinner et al., 2004). To distinguish among these possibilities, the following experiments would be necessary: (1) to identify the minimal region of the endoderm specific enhancer of *Nodal* and to examine the ectopic expression of the minimal enhancer in the *Sox17* mutants; (2) chromatin-immunoprecipitation (ChIP) with SOX17 antibodies to detect DNA binding of SOX17 in the *Nodal* genomic region; and (3) biochemical assays to determine in vivo interaction with beta-catenin and TCF factors in the definitive endoderm will be required to test the mechanisms of direct regulation of the *Nodal* PEE enhancer by SOX17.

In *Sox17* mutants, the strong expression of *Nodal-5'-LacZ* expression at the bud stages (E7.5) and early somite stages could be a consequence of higher activity of Wnt signaling in the immature endoderm cells. In support of this possibility, we have found that a Wnt signaling reporter *BAT-Gal* is expressed in the definitive endoderm at E7.5 in normal embryos. This suggests that Wnt signaling is active in these cells (data not shown). It is interesting to note that although *BAT-gal* is strongly expressed in the mesoderm, ectopic *Nodal* expression is not seen in the mesoderm in *Sox17* mutants. Wnt signals may differentially regulate definitive endoderm and mesoderm differentiation in the primitive streak, however, NODAL activity in the definitive endoderm regulated by

Wnt signaling might be specifically suppressed by SOX17 to promote differentiation of definitive endoderm cells.

Nodal signaling may contribute to defects in endoderm differentiation in *Sox17* mutants

Nodal signaling was found to be strongly active in the endoderm from 2- to 6-somite stages (E8.5) in *Sox17* mutants. How does the ectopic *Nodal* expression affect endoderm differentiation in the *Sox17* mutants? This may be explained by two factors: loss of SOX17 downstream genes, and ectopic *Nodal* expression in endoderm cells. Transverse sections of the foregut endoderm at E8.5 showed an abnormal epithelial morphology in individual cells with ectopic *Nodal* expression in *Sox17* mutant embryos, suggesting that persistent *Nodal* expression may cause delayed or block in endoderm differentiation. In our analysis of *Sox17* mutants, we found that embryos with severe foregut defects exhibited stronger ectopic *Nodal* expression in the foregut region relative to the mutant embryos with milder foregut phenotypes. This suggests that either the levels of ectopic *Nodal* expression contributes to the severity of foregut defect, or conversely, the variations in phenotype severity caused by lack of *Sox17* leads to varying degrees of ectopic *Nodal* expression. To examine if ectopic *Nodal* expression can cause defects in endoderm differentiation, overexpression studies of *Nodal* in the definitive endoderm under the control of an endoderm-specific enhancer such as *Sox17* may be useful to test the effect of excess *Nodal* on foregut differentiation in normal embryos.

Conditional deletion of *Sox17* in the entire embryo, including definitive endoderm at E6.5, leads to a weak foregut expression of the *Nodal-5'-LacZ* transgene, which contains the endoderm-specific *Nodal* enhancer. This shows that the *Nodal* expression in

emerging definitive endoderm is rapidly suppressed by SOX17, and only a few endoderm cells retain ectopic *Nodal* expression when *Sox17* function is inactivated at slightly later stages of endoderm differentiation. Although in this case, the activity of Nodal signaling pathway in the foregut in conditional *Sox17* mutants was unclear. Further experiments involving the *Lefty2-ASE* transgene and *Nodal* in situ hybridization in the conditional *Sox17* mutants at E8.5 will reveal the activation and levels of Nodal signaling in the foregut. Interestingly, we also observed a weak foregut phenotype in the conditional mutant embryos at E8.5; thus, examining the levels of Nodal signaling will reveal how expansion of NODAL signal depends on the differentiation state of the endoderm. These experiments will provide deeper insights into how *Nodal* is regulated in definitive endoderm and how ectopically expressed *Nodal* impacts endoderm differentiation in *Sox17* mutants.

Ectopic *Nodal* expression is restricted to the foregut and does not expand into the LPM in the *Sox17* mutant embryos

From our previous study, we found that *Sox17* mutants show absent or highly reduced *Nodal* expression in the LPM. However, the LPM develops normally and exogenously supplied NODAL signal in the LPM leads to activation of endogenous *Nodal* in the entire LPM (Saund et al., 2012). Thus, why is the ectopic *Nodal* expression in the foregut endoderm cells unable to induce *Nodal* expression in the LPM in the *Sox17* mutants? Both *Nodal* and *Lefty2* are expressed in discrete cells in the foregut in the mutant embryos; however, *Pitx2* is more broadly expressed, likely due to the higher relative activity of the ASE than *Nodal* and *Lefty2*. These observations indicate that although *Nodal* ectopic expression does not expand in the entire foregut, Nodal signaling

is pervasive in the foregut endoderm in *Sox17* mutants, suggesting that NODAL is secreted broadly within the foregut endoderm.

If NODAL is secreted in the endoderm layer, why does ectopic *Nodal* expression in endoderm cells fail to induce *Nodal* in the LPM in *Sox17* mutants even though the *Nodal* expressing endoderm cells are localized adjacent to the LPM cells? Although NODAL signals easily spread to neighboring endoderm cells in the *Sox17* mutants, the ECM barrier in the basal region of the endoderm layer and lack of cell polarity in the *Sox17* endoderm cells may prevent NODAL proteins from secreting into the lateral plate mesoderm region, even though the components required for Nodal signaling are present in these cells (Saund et al., 2012).

Materials and methods

Mice and embryo collection

Heterozygous *Sox17* null and $Sox17^{GFP}$ mice were maintained in a mixed 129sv, C57BL/6J and CD-1, and 129sv and C57BL/6J background, respectively. The homozygous $Sox17^{F/F}$ mice were maintained in the C57BL/6J background. The homozygous *RosaCreER,* and *dTdG* mice were maintained in the 129sv and C57BL/6J background. The *Lefty2-ASE* and *Nodal-5'-LacZ* mice were obtained from the lab of Dr. Hiroshi Hamada (Osaka University) and maintained in the 129sv/C57BL/6J and CD-1 mixed background. The pregnant females were monitored by ultrasound scan (Visual Sonics) to estimate the stage of embryos prior to harvest. Genotyping of embryos was performed according to established methods described previously. The Institutional Animal Care and Use Committee (IACUC) protocol was followed for all procedures for the maintenance of mice at The University of Utah.

Whole mount in situ hybridization and X-gal staining

Whole mount in situ hybridization for *Nodal* was performed according to standard procedures (Wilkinson, 1998) using a combination of three plasmids containing 5′ and 3′ UTR and the coding sequence. Following whole-mount in situ hybridization, embryos were imaged in 80% glycerol and 10 μm sections were prepared by standard paraffin sectioning. For X-gal staining, embryos were fixed for 15 minutes in 0.8% PFA, 0.25% glutaraldehyde in PBN (PBS containing 0.01% NP-40), washed three times in PBN and incubated in staining solution containing X-gal (0.2mg/ml) at 37^0C until beta-galactosidase activity developed they were then fixed in 4% PFA for 15 minutes and stored.

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References

Adachi, H., Saijoh, Y., Mochida, K., Ohishi, S., Hashiguchi, H., Hirao, A. and Hamada, H. (1999). Determination of left/right asymmetric expression of nodal by a left side-specific enhancer with sequence similarity to a lefty-2 enhancer. *Genes Dev* **13**(12), 1589-600.

Arnold, S. J. and Robertson, E. J. (2009). Making a commitment: cell lineage allocation and axis patterning in the early mouse embryo', *Nat Rev Mol Cell Biol* **10**(2), 91-103.

Ben-Haim, N., Lu, C., Guzman-Ayala, M., Pescatore, L., Mesnard, D., Bischofberger, M., Naef, F., Robertson, E. J. and Constam, D. B. (2006). The nodal precursor acting via activin receptors induces mesoderm by maintaining a source of its convertases and BMP4. *Dev Cell* **11**(3), 313-23.

Conlon, F. L., Lyons, K. M., Takaesu, N., Barth, K. S., Kispert, A., Herrmann, B. and Robertson, E. J. (1994). A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. *Development* **120**(7), 1919-28.

Costello, I., Pimeisl, I. M., Drager, S., Bikoff, E. K., Robertson, E. J. and Arnold, S. J. (2011). The T-box transcription factor Eomesodermin acts upstream of Mesp1 to specify cardiac mesoderm during mouse gastrulation. *Nat Cell Biol* **13**(9), 1084-91.

Gadue, P., Huber, T. L., Paddison, P. J. and Keller, G. M. (2006). Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. *Proc Natl Acad Sci USA* **103**(45), 16806-11.

Hallonet, M., Kaestner, K. H., Martin-Parras, L., Sasaki, H., Betz, U. A. and Ang, S. L. (2002). Maintenance of the specification of the anterior definitive endoderm and forebrain depends on the axial mesendoderm: a study using HNF3beta/Foxa2 conditional mutants. *Dev Biol* **243**(1), 20-33.

Hamada, H., Meno, C., Watanabe, D. and Saijoh, Y. (2002). Establishment of vertebrate left-right asymmetry. *Nat Rev Genet* **3**(2), 103-13.

Kanai-Azuma, M., Kanai, Y., Gad, J. M., Tajima, Y., Taya, C., Kurohmaru, M., Sanai, Y., Yonekawa, H., Yazaki, K., Tam, P. P. et al. (2002). Depletion of definitive gut endoderm in Sox17-null mutant mice. *Development* **129**(10), 2367-79.

Kwon, G. S., Viotti, M. and Hadjantonakis, A. K. (2008). The endoderm of the mouse embryo arises by dynamic widespread intercalation of embryonic and extraembryonic lineages. *Dev Cell* **15**(4), 509-20.

Liu, X., Luo, M., Xie, W., Wells, J. M., Goodheart, M. J. and Engelhardt, J. F. (2010). Sox17 modulates Wnt3A/beta-catenin-mediated transcriptional activation of the Lef-1 promoter. *Am J Physiol Lung Cell Mol Physiol* **299**(5), L694-710.

Liu, Y., Asakura, M., Inoue, H., Nakamura, T., Sano, M., Niu, Z., Chen, M., Schwartz, R. J. and Schneider, M. D. (2007). Sox17 is essential for the specification of cardiac mesoderm in embryonic stem cells. *Proc Natl Acad Sci USA* **104**(10), 3859-64.

Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L. and Luo, L. (2007). A global double-fluorescent Cre reporter mouse. *Genesis* **45**(9), 593-605.

Norris, D. P., Brennan, J., Bikoff, E. K. and Robertson, E. J. (2002). The Foxh1-dependent autoregulatory enhancer controls the level of Nodal signals in the mouse embryo. *Development* **129**(14), 3455-68.

Norris, D. P. and Robertson, E. J. (1999). Asymmetric and node-specific nodal expression patterns are controlled by two distinct cis-acting regulatory elements. *Genes Dev* **13**(12), 1575-88.

Park, E. J., Sun, X., Nichol, P., Saijoh, Y., Martin, J. F. and Moon, A. M. (2008). System for tamoxifen-inducible expression of cre-recombinase from the Foxa2 locus in mice. *Dev Dyn* **237**(2), 447-53.

Perea-Gomez, A., Vella, F. D., Shawlot, W., Oulad-Abdelghani, M., Chazaud, C., Meno, C., Pfister, V., Chen, L., Robertson, E., Hamada, H. et al. (2002). Nodal antagonists in the anterior visceral endoderm prevent the formation of multiple primitive streaks. *Dev Cell* **3**(5), 745-56.

Saijoh, Y., Adachi, H., Sakuma, R., Yeo, C. Y., Yashiro, K., Watanabe, M., Hashiguchi, H., Mochida, K., Ohishi, S., Kawabata, M. et al. (2000). Left-right asymmetric expression of lefty2 and nodal is induced by a signaling pathway that includes the transcription factor FAST2. *Mol Cell* **5**(1), 35-47.

Saijoh, Y., Oki, S., Ohishi, S. and Hamada, H. (2003). Left-right patterning of the mouse lateral plate requires nodal produced in the node. *Dev Biol* **256**(1), 160-72.

Saund, R. S., Kanai-Azuma, M., Kanai, Y., Kim, I., Lucero, M. T. and Saijoh, Y. (2012). Gut endoderm is involved in the transfer of left-right asymmetry from the node to the lateral plate mesoderm in the mouse embryo. *Development* **139**(13), 2426-35.

Shiratori, H., Sakuma, R., Watanabe, M., Hashiguchi, H., Mochida, K., Sakai, Y., Nishino, J., Saijoh, Y., Whitman, M. and Hamada, H. (2001). Two-step regulation of left-right asymmetric expression of Pitx2: initiation by nodal signaling and maintenance by Nkx2. *Mol Cell* **7**(1), 137-49.

Sinner, D., Rankin, S., Lee, M. and Zorn, A. M. (2004). Sox17 and beta-catenin cooperate to regulate the transcription of endodermal genes. *Development* **131**(13), 3069-80.

Tremblay, K. D., Hoodless, P. A., Bikoff, E. K. and Robertson, E. J. (2000). Formation of the definitive endoderm in mouse is a Smad2-dependent process. *Development* **127**(14), 3079-90.

Vincent, S. D., Dunn, N. R., Hayashi, S., Norris, D. P. and Robertson, E. J. (2003). Cell fate decisions within the mouse organizer are governed by graded Nodal signals. *Genes Dev* **17**(13), 1646-62.

Woodland, H. R. and Zorn, A. M. (2008). The core endodermal gene network of vertebrates: combining developmental precision with evolutionary flexibility. *Bioessays* **30**(8), 757-65.

CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

Summary

In this thesis, we have reported a novel role for endoderm cells in transferring LR signal information from the node to the LPM. So far, all events that have been reported in LR determination have involved mesodermal cells such as the node, LPM, notochord/floor plate and somites. In *Sox17* null mutants, with defects specifically in the defective endoderm, there is an absence or reduction of the normal expression of LR asymmetric genes in the LPM. The node shape is affected due to the presence of isolated endoderm cells that fail to clear from the node region; however, LR determination across the node occurs normally as evidenced by the relatively normal leftward nodal flow and the asymmetric expression of perinodal genes. The LPM in *Sox17* mutants is responsive to the activation of endogenous *Nodal* expression when supplied with an exogenous source of *Nodal* directly into the LPM. Therefore, the loss or reduction in asymmetric gene expression in the LPM in *Sox17* mutants most likely occur in the process of signal transfer from the node to the LPM.

Our analysis of connexin protein distribution and physiological assays of gap junctional gating in *Sox17* mutant embryos suggest that gap junctional communication between endoderm cells plays an important role in the process of the LR signal transfer. In control embryos, we found that CX43 proteins were expressed in the cell membrane in

both extraembryonic and embryonic endoderm cells but not in the node. In *Sox17* mutant embryos, CX43 was weakly expressed in the endoderm cells in the embryonic region, but normally localized to the membrane in extraembryonic endoderm. Gap junction permeability between endoderm cells was tested by iontophoresis of a gap junction permeable dye, Lucifer Yellow, into single endoderm cells and found to be severely compromised in the embryonic endoderm region in *Sox17* mutants. Impaired gap junction communication between endoderm cells in *Sox17* mutants may be due to defects in the differentiation of endoderm cells. We then examined the possibility that *Sox17* mutant endoderm formed cell-cell contacts between adjacent endoderm cells. The tight junction component ZO1 was found to be localized on the cell membrane of *Sox17* mutant endoderm cells similar to control endoderm, suggesting that *Sox17* mutant endoderm layer has an epithelial structure that develops tight junctions. Further studies of cellular morphology using a $Sox17^{GFP/GFP}$ mutant allele, that expresses GFP only in endoderm under the control of endogenous *Sox17* transcriptional regulation, showed loss of epithelial polarity in mesenchymal-like shaped cells that were delaminated from the epithelial layer. The lack of structural rigidity due to abnormal cytoskeletal assembly together with disorganized adherens junctions explain the differentiation defects in endoderm cells of *Sox17* mutants. These defects in epithelial morphology may result in the loss of gap junctional communication by both lower transcription levels of *Cx43* and reduced localization of Cx43 on the cell membrane.

Previously, it was reported that proteoglycans with glucosaminoglycan moieties such as chondroitin sulfate and heparan sulfate play important roles in transfer of LR signals from the node to the LPM to induce *Nodal* expression in the left LPM (Oki et al.,

2007). Chondroitin sulfate is distributed at the interface between endoderm and mesoderm cells in control embryos and was shown to directly interact with NODAL protein (Oki et al., 2007). We showed that 40% of *Sox17* mutant embryos (7 of 17) showed mild regional defects in chondroitin sulphate distribution and laminin matrix disorganization, whereas the remaining mutant embryos showed almost normal distribution. Since the gap junctional defect and the proportion of *Sox17* mutants showing LR defects (90% embryos with absent *Nodal* expression in LPM) is more severe than defects in proteoglycan distribution, the major cause of the LR phenotype in *Sox17* mutants seems to be the loss of gap junction function in the endoderm.

How does gap junctional communication through endoderm cells near the node mediate signal transfer from the node to the LPM? Gap junctions have been reported to function in LR determination in several animal models, such as chick, Xenopus, zebrafish and rabbit (Feistel and Blum, 2008; Hatler et al., 2009; Levin and Mercola, 1998; Levin and Mercola, 1999). Our study has shed light on the role of gap junctions in LR asymmetry in mouse embryos in a different context compared to these animals. The following proposed models reveal possible mechanisms by which endoderm cells mediate the passage of LR information from the node to the LPM.

Models for role for endoderm in LR determination

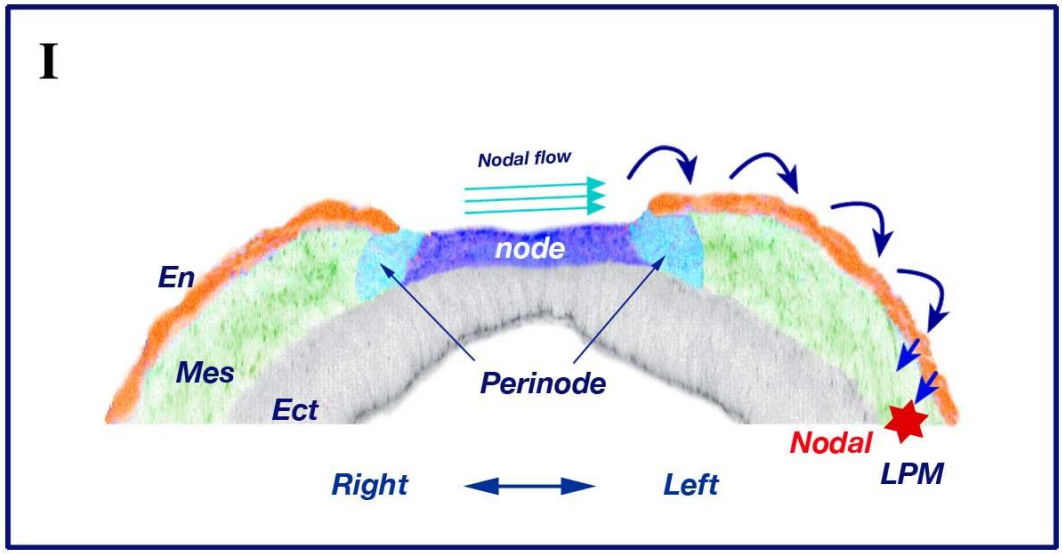
Our data clearly establishes that endoderm plays an essential role in the transfer of LR signal from the node to the LPM. The endoderm forms a continuous layer extending from the crown cells of the node, that partially underlie the endoderm layer, until the LPM, which is completely covered by the endoderm. Thus, what are the likely LR signal transfer mechanisms from the node to the LPM that are mediated by the endoderm?

There are two possibilities based on the current results and previous studies on LR signal transfer: (1) Direct relay of LR signal information via gap junctions through successive endoderm cells from the node to the LPM. (2) Local amplification of the LR signal received on the left side of the node to allow accumulation of processed NODAL protein in the perinode cells that diffuses through the mesoderm layer to the LPM.

Model I: Direct relay of left-right signal via the endoderm layer from the node to the LPM

The endoderm layer between the node and the LPM exists as a planar sheet of cells, separated from the mesoderm cells by a basement membrane (Figure 5.1). The gap junctions among endoderm cells provide a mode of intercellular communication that allows rapid propagation of second messenger molecules within the endoderm layer. Our results show that injection of the gap junction permeable dye Lucifer Yellow into single endoderm cells of normal embryos at 4-somite stage leads to rapid transfer into neighboring endoderm cells (Chapter 3, Figure 3.6G). In *Sox17* mutants, the gap junctional communication between endoderm cells was severely compromised, where the injected dye either remained in the injected cell or spread weakly into a neighboring cell (Chapter 3, Figure 3.6H). The importance of gap junction communication in LR determination was also shown by Hadjantonakis and colleagues, using a general connexin inhibitor, 18 alpha-glycyrrhetinic acid to inhibit gap junctions in mouse embryos growing in culture (Viotti et al., 2012). A majority of wildtype embryos cultured from the headfold to the 4-somite stage exhibited absent asymmetric gene expression in the LPM and the remaining embryos showed reduced left sided expression. Here, although the target tissue of 18 alpha-glycyrrhetinic acid was not identified as endoderm, our

Figure 5.1 Proposed models for the role of endoderm in transfer of signals from the node to the left LPM. Model I: Signal relay through endoderm cells via gap junction communication. After nodal flow is established, the LR signal generated in the node reaches endoderm cells. Then the signal is transferred by a relay of small molecules (navy blue arrows) such as Ca^{2+}, through gap junctions and activate the left LPM cells to induce *Nodal* expression (red star). Model II: Modification of perinode cell function to promote NODAL protein secretion and transport to the LPM. After endoderm receives the LR signal that is determined by nodal flow, the signal expands in a small region of endoderm cells (orange) near the node that covers perinode cells (light blue, navy arrows). The active NODAL protein complex is formed in the left perinode cells due to interaction with the overlying endoderm, which promotes transfer (pink arrows) of the activated NODAL (red dots) through the extracellular matrix in mesoderm or between endoderm and mesoderm to induce *Nodal* expression in the left LPM (red star). NODAL that is expressed in the right perinode cells is not activated (blue dots). Ect: ectoderm (gray), En: endoderm (orange), Mes: mesoderm (green).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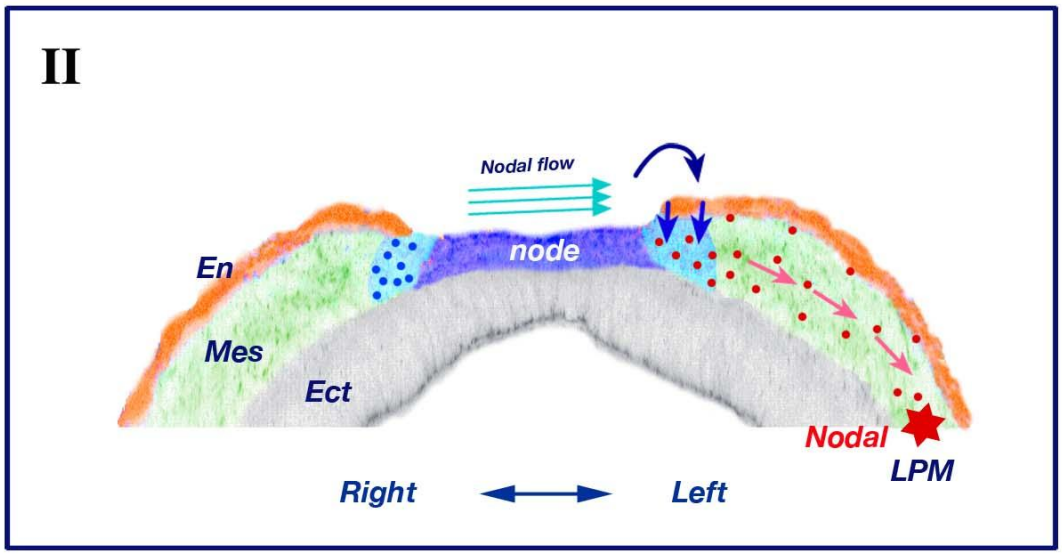
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Left

immunohistochemical studies showed that Cx43 was most strongly expressed in the endoderm cell membrane at the corresponding stage, suggesting that gap junctional communication occurs predominantly in endoderm cells (Chapter 3, Figure 3.6A). In *Sox17* mutants, the endoderm layer fails to maintain epithelial polarity and exhibits disorganized adherens junction between neighboring endoderm cells. *Cx43* mRNA expression levels and membrane protein localization in *Sox17* mutants were considerably lower compared to control embryos (Chapter 3, Figure 3.5).

How does endoderm communicate through gap junctions to relay LR signal to activate left-sided gene expression in the LPM? We have shown that in *Sox17* mutants, the midline tissues formed normally and nodal flow was detected in the leftward direction over the node surface, suggesting that the symmetry breaking occurs in the normal orientation (Chapter 3, Figure 3.3). Although the nature of the asymmetric signal generated at the left side of the node is not understood, Ca^{2+} flux on the left endoderm has been observed by several groups either proximal or distal to the node margin (Hadjantonakis et al., 2008; McGrath et al., 2003; Song et al., 2010; Tanaka et al., 2005), suggesting a possible mechanism involving Ca^{2+} mediated signal transfer in endoderm cells. In this model, the gap junctional communication between endoderm cells functions to passively transmit the Ca^{2+} signal from the node periphery to the region covering the lateral plate mesoderm.

Although the correlation between elevated Ca^{2+} in the left side endoderm and *Nodal* expression in the left LPM has not been examined in the mouse, a potential role of Ca^{2+} in LR determination has been reported in zebrafish (Francescatto et al., 2010). CaMK-IIalpha is activated transiently in surrounding cells of the Kuppfer's vesicle

(corresponds to the node of mouse embryos) only on the left side in a similar pattern to the Ca^{2+} flux in zebrafish. Morpholino knockdown experiments of *CamK-II* genes resulted in loss of *Nodal* gene expression in the LPM in 20-30% of embryos examined. This result suggests that the calcium flux may mediate LR determination to induce the left side expression of *Nodal* in the LPM.

In this model, the endoderm layer serves as the principal conduit to transfer the asymmetric signal from the node upto the LPM; however, it is possible that the perinode cells, which are critical to receive the LR signal from nodal flow may be involved in the upregulation of Ca^{2+} flux in the endoderm cells. Thus far, elevated Ca^{2+} levels in endoderm is the only LR asymmetric signal observed between the node and the LPM, further studies will be required to test whether the Ca^{2+} signal in endoderm is sufficient to activate *Nodal* expression in the LPM.

Model II: Modification of perinode cell function to promote NODAL protein secretion and transport to the LPM

The endoderm cells lining the edge of the node periphery directly overlie a subset of perinode cells that remain unexposed, while the remaining perinode cells have projecting cilia (Chapter 3, supplementary figure S3.2). Recent studies have shown that the cilia on perinode cells act as sensors of mechanical force generated by nodal flow, through uptake of free calcium by PKD2 receptors on the perinodal cilia (Yoshiba et al., 2012). Free intracellular calcium ions have been observed in endoderm between the node and the lateral plate mesoderm at the early somite stages. Higher levels of free calcium ions were seen in the endoderm cells lining the node margin, presumably in direct contact with the underlying perinode cells (McGrath et al., 2003; Song et al., 2010; Tanaka et al.,

2005). These data suggest a close interaction between the perinode and the peripheral endoderm that may result in increased free calcium signal in response to directional nodal flow.

Nodal is expressed asymmetrically in the perinode cells, higher on the left side from 3- to 6-somite stages and shown to be required for the expression of *Nodal* in the left LPM (Saijoh et al., 2003). The NODAL antagonist CERL-2 directs the correct orientation of the signal to the left LPM (Marques et al., 2004), suggesting the importance of "the active NODAL signal" for transfer of the signal from the node to the LPM. Since higher Ca^{2+} flux is observed in the left endoderm near the node, these endoderm cells may modify the process of active NODAL complex formation. A recent paper reported that the Nodal signaling mediated by phospho-SMAD2/3 is activated only in the left perinodal cells in normal embryos (Kawasumi et al., 2011). However, the inhibition of the phospho-SMAD2/3 activity by transgenic expression of dominant-negative transgenic pSMAD3 in the node does not repress *Nodal* expression in the left LPM. Hence, the secretion of NODAL in the perinode cells, but not Nodal signaling activity in the perinodal cells, appears to be necessary to induce *Nodal* in the LPM. In the same study, the higher expression of *Cerl-2* in the right side of node preceded asymmetric pSMAD2/3 signaling in the perinode. *Sox17* mutant embryos have a smaller node size, but contain similar numbers of cilia compared to normal embryos that are capable of generating leftward nodal flow including sensory perinodal cilia. *Nodal* and *Cerl-2* are expressed and show asymmetric expression patterns in the perinode region of *Sox17* mutants similar to normal embryos (Chapter 3, Figure 3.3). This suggests that defects in the interaction of endoderm with the perinode subsequent to the establishment

of *Cerl-2* asymmetry in the node may be responsible for the loss of transfer of signal in the mutant embryos.

The transfer of NODAL from the node to the LPM was shown to depend on sulfated glycosaminoglycans, chondriotin and heparan sulfates that are localized at the basement membrane underneath the endoderm layer and in the interstitial space between mesoderm cells (Oki et al., 2007). In *Sox17* mutants, the distribution of chondroitin sulfate and the extracellular matrix component, laminin in the mesoderm was only partially affected and not the cause of LR defects (Chapter 3, Figure 3.4). This suggests that the LR asymmetry across the node is normal but the signal fails to be delivered to the LPM in the *Sox17* mutants. Thus, according to this model, the endoderm defects resulted in deficient processing of NODAL in the perinode cells leading to reduced availability of secreted NODAL to traverse across the mesoderm to the LPM in *Sox17* mutants.

Preferred model for LR signal transfer

In the preceding section, two models have been proposed for the mechanisms by which endoderm plays a role in mediating the relay of LR signal information from the node to the LPM. In the first model, the LR signal on the left side of the node is transferred via gap junctions in the endoderm directly to the LPM, leading to activation of Nodal signaling pathway (Figure 5.1). Although several groups have observed free intracellular calcium signal in the endoderm layer using Fluo-4 dye by confocal imaging, the higher levels of calcium were observed in endoderm cells either near the node or distant from the node (McGrath et al., 2003; Song et al., 2010; Tanaka et al., 2005). It is likely that the calcium transfer between cells is very rapid and more sensitive techniques will be required to test whether calcium signal flows from the node outwards to the LPM.

Also, it is not clear how calcium signal in the endoderm over the LPM may directly lead to activation of *Nodal* expression in the LPM, since these phenomena occur in separate cell layers and there are no reported calcium sensitive enhancers in the *Nodal* genomic region.

A spate of recent studies have provided new insights into the mechanisms by which calcium signaling may play a role in LR determination. Hamada and coworkers showed that the calcium ion channel PKD2, localized in the cilia in perinodal cells is required for sensing the nodal flow and is essential for LR patterning. This provides evidence for the two-cilia theory, first proposed by Tabin and colleagues (Tabin and Vogan, 2003; Yoshiba et al., 2012). Using Sox17-GFP knock-in embryos that express GFP specifically in the endoderm layer during the early somite stages when nodal flow is active, we have shown that the endoderm cells lie in immediate contact and directly over the perinodal cells (Chapter 3, supplementary figure S3.3). Thus, it is very likely that the calcium signal is transduced from the perinode to the endoderm cells and propagates within the endoderm layer through gap junctions. Since the signal originates at the perinodal cells and is continuously generated by the leftward nodal flow, the calcium signal is likely to be strongest in the endoderm cells at the node margin as revealed by several studies (McGrath et al., 2003; Song et al., 2010; Tanaka et al., 2005). However, it is not yet understood how the calcium signal in the perinode and the endoderm integrates with the overall scheme of LR determination. Using inhibitors of IP3 and ER-ATPase receptors that abolished release of free calcium ions from intracellular stores, Yoshiba et. al., (2012) have shown that the LR symmetry breaking in the perinode was impaired, suggesting that the site of action of the calcium signaling appears to be perinode cells.

Although free intracellular calcium ions were not previously observed within the perinode by Fluo3/4 dyes, a recent study using Fura-2 calcium indicator dye, has demonstrated that perinode cells also show calcium ion elevations dependent on the direction of nodal flow and the PKD2 receptor (Takao et al., 2013). The authors also show that pharmacological perturbation using ionomycin, an inhibitor that increases intracellular levels of calcium signal, resulted in bilateral *Nodal* expression in the LPM concomitant with equal expression of *Cerl-2* in both sides of the perinode. This suggests that the perinode is a major target of calcium signaling. Since *Pkd2* acts as a calcium sensor at the perinode cilia, the relationship between *Cerl2* and the *Pkd2* has been further investigated to understand how calcium signaling may affect LR asymmetry in the node (Yoshiba et al., 2012). In normal embryos *Cerl-2* expression in the perinode is higher in the right side, in a manner dependent on nodal flow, whereas in *Cerl-2* mutants, *Nodal* expression is randomized in the LPM (Marques et al., 2004). In *Pkd2* null mutants, which are unable to sense nodal flow and generate elevated calcium ions levels both in the perinode and the endoderm, *Cerl-2* was expressed equally on both sides in the perinode and *Nodal* expression is absent in the LPM (McGrath et al., 2003; Takao et al., 2013; Yoshiba et al., 2012). However, in *Cerl-2* and *Pkd2* double mutants, *Nodal* expression is randomized in the LPM similar to *Cerl-2* mutants, suggesting that *Cerl-2* functions downstream of *Pkd2* in the perinode (Yoshiba et al., 2012). Thus, there is accumulated evidence that supports the notion that the PKD2-dependent calcium signal produced in response to the nodal flow acts locally to regulate the asymmetric expression of *Cerl-2* and *Nodal* in the perinode.

In model II, we have proposed that subsequent to leftward nodal flow, endoderm cells at close proximity to the node provide signaling cues to the perinode to promote the activation and release of the NODAL protein complex that is then relayed to the LPM, establishing left-sided gene expression patterns. The higher levels of free calcium ions in the endoderm adjoining the node are functionally dependent on the PKD2 receptor in the perinodal cilia. This suggests a possible link between activation of calcium signaling pathways in the endoderm and the mechanisms regulating the asymmetric expression of *Cerl-2* and *Nodal* in the perinode. There is considerable interest in uncovering how nodal flow is translated to generate asymmetry across the node and further research will shed new insights into the precise role of calcium signaling in the perinode region. Development of new antibodies against NODAL and the antagonist CERL-2 will aid in understanding how the dynamics of LR asymmetry in the perinode are established at the protein level in response to nodal flow. Future studies will reveal further insights into how calcium signaling in the endoderm layer is integrated in the mechanisms that generate robust asymmetric signal, in accordance with model II.

Future directions - Identity and function of intercellular messengers through gap junctions in endoderm

The nature of the asymmetric LR signal generated in the node in response to nodal flow is, so far, unknown. Our studies clearly show the involvement of gap junctions in the establishment of LR asymmetry. A candidate for gap junctional transport through endoderm cells is free calcium ion, Ca^{2+}. The aforementioned models for the role of definitive endoderm suggest that free Ca^{2+} may be required either at the LPM to

activate LR asymmetric gene expression or at the perinode to regulate the release of active NODAL, which is transported to the LPM to initiate *Nodal* expression.

What are the possible methods to test the requirement of free calcium ions in the activation of asymmetric gene expression in the LPM?

Firstly, the IP3 receptors are involved in the cytoplasmic release of intracellular stores of free calcium ions, and shown to be necessary for LR determination (Sarmah et al., 2005; Yoshiba et al., 2012). It is not clear whether specific inhibition of calcium flux in the endoderm in normal mouse embryos is sufficient to affect LR asymmetric gene expression in the LPM. Treatment of wildtype embryos with an inhibitor of IP3 receptor, such as 2- aminoethoxydiphenyl borate (2-APB) may be used to test the dependence of asymmetric gene expression in the LPM on calcium ion flow through endoderm.

Secondly, in another approach, Viotti et al., showed that the general inhibition of gap junctions in whole embryos led to absent or reduced LR gene expression in the LPM (Viotti et al., 2012). The gap junction communication through endoderm may be required locally near the node or more distantly in the LPM for the induction of LR information. Although in *Sox17* mutants the endoderm shows severely reduced gap junctional transport concomitant with LR gene expression in the LPM, the defects in endoderm may affect release of the active NODAL protein complex from the perinode, or directly affect *Nodal* expression in the LPM. Inhibition of gap junctions by 18 alpha-glycyrrhetinic acid (AGA) or heptanol in whole embryo culture, followed by examination of LR asymmetry across the node and the LPM in the same embryos will be useful to test this possibility.

Thirdly, the inhibition of gap junctional communication by chemical inhibitors in culture is limited by the toxicity and insufficient efficacy on target connexins. The

transgenic expression of a dominant-negative form of CONNEXIN43, the major connexin expressed in the endoderm, can be used to overcome this problem (Banks et al., 2009; Beahm et al., 2006). Particularly, the mutant CX43-G138R localizes to the membrane and causes failure of electrical coupling with wild type CX43 via a dominant negative effect in Xenopus oocytes and cultured cells (Dobrowolski et al., 2008). The genetic approach of conditional activation of floxed CX43 dominant-negative forms will allow *in vivo* testing of the requirement of gap junctional coupling for the precise step of LR signal transfer between the node and the LPM.

Finally, visualization of the active NODAL protein complex as well as activation of Nodal signaling by pSMAD2/3 phosphorylation by immunohistochemical methods between the node and the LPM will further aid the investigation of the role of the gap junctional transport on the transfer of LR information to the LPM. These studies will elaborate the models of the passage of LR signal transfer subsequent to nodal flow to the LPM.

SOX17 regulates *Nodal* expression in the foregut endoderm

Our data clearly show that *Sox17* mutant embryos have defects in endoderm differentiation in the embryonic region. Surprisingly, we observed that endoderm cells in the foregut region of *Sox17* mutant embryos continued to express *Nodal* at early somite stages. *Nodal* is an upstream regulatory factor that induces endoderm cells during gastrulation but the expression is immediately shut down in differentiated endoderm. We predicted that the continued expression of *Nodal* maintains the definitive endoderm cells in an immature state in *Sox17* mutant embryos and prevents the cells from differentiating

and proliferating normally (Figure 5.2). At the late streak stage, *Nodal* expression was reduced in the endoderm layer by the early bud stage. In the *Sox17* mutant embryos, migrating endoderm cells still continued to express *Nodal* at the bud stage (Chapter 4, Figure 4.1). Thus, the ectopic expression of *Nodal* in *Sox17* mutant embryos appears to be caused by the failure to downregulate transcription at an early stage of endoderm differentiation.

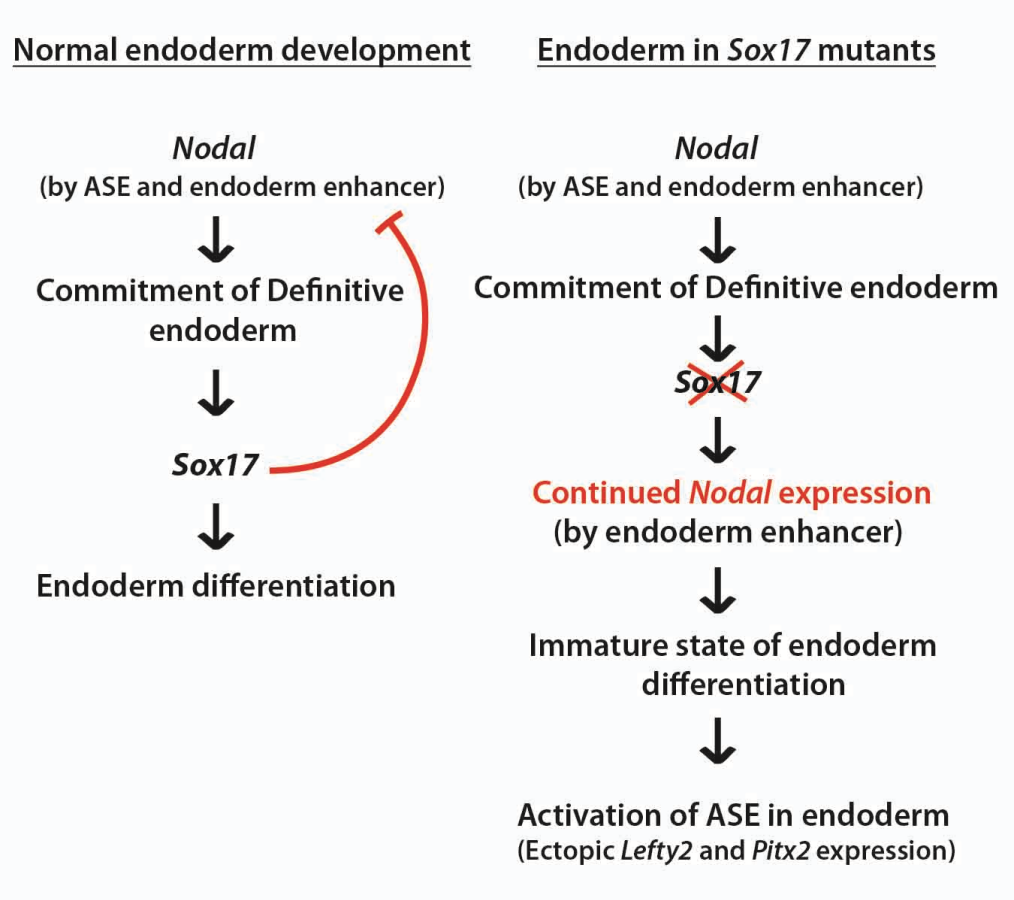

Figure 5.2 Model for regulation of the *Nodal* expression and activity by SOX17 in differentiating endoderm cells.

Nodal signaling pathway is activated in the mutant foregut during late gastrulation

We then investigated the mechanism controlling *Nodal* transcriptional regulation during endoderm differentiation. At early somite stages, downstream genes of *Nodal*, *Lefty2* and *Pitx2* were also ectopically expressed in the foregut region similar to *Nodal* (Chapter 3, Figure 3.1). This suggests that the transcriptional feedback loop of NODAL observed in the left LPM is active in endoderm cells of *Sox17* mutant embryos. To examine this possibility, a reporter transgene for *Nodal* feedback regulation, *Lefty2 ASE-lacZ* (Saijoh et al., 1999) was introduced into *Sox17* mutants. At early somite stages, the *Lefty2 ASE* activity was obvious in the foregut endoderm similar to *Nodal*, *Lefty2* and *Pitx2* expression in the *Sox17* mutant embryos, whereas the control embryos expressed the reporter gene only in the left LPM and the floor plate (Chapter 4, Figure 4.2B).

In normal development, the reporter activity that was abundant in the primitive streak at the streak stage was attenuated by the bud stage. In the *Sox17* mutant embryos, the expression pattern was similar to the control embryos and no ectopic activity was observed in the endoderm cells at these stages (Chapter 4, Figure 4.2A). This indicates that in the *Sox17* mutant embryos, ectopic *Nodal* expression in endoderm cells is regulated by a positive and negative feedback loop of *Nodal* transcription at the early somite stage, however, this *Nodal* feedback regulation is not active at the earlier stage of endoderm differentiation.

A novel endoderm-specific enhancer of *Nodal* is responsible for the ectopic expression during early gastrulation stages

We predicted that *Nodal* has another enhancer that is responsible for expression in the definitive endoderm cells as they migrate out from the primitive streak. We found that a *Nodal* transgene that contains a 13 kb fragment, upstream of the *Nodal* transcriptional start site linked to a *LacZ* reporter (Adachi et al., 1999), was active in the migrating endoderm cells at the streak stage in control embryos. However, the activity of the reporter gene becomes lower at the bud stage and was not observed at somite stages. This suggests that the 5'-upstream region contains an enhancer that is responsible for *Nodal* expression in migrating endoderm cells from the primitive streak and that it is distinct from the *ASE*. In the *Sox17* mutant embryos, the transgene was also expressed at the streak stage and the activity continued through the early somite stage similar to the ectopic expression of the endogenous *Nodal* gene (Chapter 4, Figure 4.2C, D). These transgenic analyses suggest that an important role of SOX17 in endoderm differentiation is to turn off *Nodal* expression to promote immature mesoendoderm cells to differentiate into endoderm with defined epithelial structures; this likely allows the transfer of LR signal from the node via gap junction communication.

SOX17 may suppress *Nodal* expression during early gastrulation by negative regulation of Wnt signaling

Ectopic expression of *Nodal* in the foregut endoderm in *Sox17* mutants is regulated by two different enhancers: the *Nodal* endoderm enhancer that is localized within a 13 kb upstream region of the *Nodal* gene and a LR asymmetric enhancer, ASE responsible for NODAL positive feedback regulation. Investigation of the 13kb upstream

region has previously revealed that the proximal enhancer element (PEE) is essential for the initiation of *Nodal* expression in the vicinity of the extraembryonic ectoderm at E6.0 using PEE-LacZ transgenic embryos (Norris and Robertson, 1999). It was also found that the PEE-LacZ transgene expression in the epiblast was lost in *Wnt3* homozygous null mutants (Ben-Haim et al., 2006; Norris and Robertson, 1999). This suggests that the initiation of *Nodal* expression in the epiblast may depend on canonical Wnt signaling via the PEE. Evidence for direct regulation of *Nodal* transcription by Wnt signaling comes from the detection of two close-lying highly conserved TCF binding sites in the PEE (Ben-Haim et al., 2006). However, direct binding of TCF to these sites has not been shown yet. These evidences suggest that Wnt signaling may directly regulate the expression of *Nodal* expression in the epiblast. Canonical Wnt signaling member *Wnt3* is expressed in the primitive streak, and β-CATENIN also appears to be localized to the nucleus in the newly migrating endoderm cells from the primitive streak (Mohamed et al., 2004).

Previous reports suggest that SOX17 interacts with canonical Wnt pathway to negatively regulate expression of target genes (Sinner et al., 2004). Direct physical interaction between mouse SOX17 and β-CATENIN has been shown in a cell culture system, indicating β-CATENIN may act as a cofactor for the SOX17 transcription factor (Liu et al., 2007). Our studies on the expression of Wnt signaling responsive BAT-GAL transgene in the migrating endoderm cells reveal active Wnt canonical signaling in the definitive endoderm derived from the primitive streak (data not shown). Taken together, these observations suggest that once *Sox17* is expressed in the newly formed definitive endoderm cells migrating from the primitive streak, it interferes with the PEE dependent

activation of *Nodal* transcription by Wnt signaling to suppress *Nodal* expression in these cells. Thus, in the absence of SOX17, *Nodal* continues to be expressed in the endoderm cells from the primitive streak stage to the somite stage.

Future directions to test the functional significance of ectopic *Nodal* expression

How does *Nodal* ectopic expression in endoderm cells affect LR determination? It has been shown that endoderm specification in the primitive streak requires higher NODAL activity than mesoderm, as evidenced by the analysis of hypomorphic *Nodal* mutants and other model animals (Schier and Shen, 2000). After endoderm cells differentiate and migrate away from the primitive streak, *Nodal* expression in these cells ceases immediately. Several studies have suggested that downregulation of NODAL activity is essential for endoderm differentiation (Meno et al., 1999; Thisse and Thisse, 1999). Thus, continued expression of *Nodal* may contribute to the endoderm defects in the *Sox17* mutants. To test this possibility, *Nodal* expression in the definitive endoderm may be induced under the control of an endoderm specific promoter in normal embryos. The activity of a *Lefty1* enhancer in definitive endoderm at the presomite stages is similar to the *Sox17* expression pattern (data not shown), potentially allowing for ectopic *Nodal* expression in wildtype endoderm cells. Another approach is the repression of the ectopic *Nodal* expression in *Sox17* mutants, by forced expression of *Lefty2*, an inhibitor of Nodal signaling, in the endoderm cells. For this purpose, the *Nodal* endoderm enhancer previously shown to be ectopically expressed in *Sox17* mutants, similar to *Nodal* expression, will be useful (Chapter 4, Figure 4.2). The higher expression levels of *Lefty2* in the endoderm will result in a decreased level of Nodal signaling due to negative

feedback regulation, in a manner similar to the induced expression of *Lefty2* in the perinode to suppress the NODAL signal at the node (Saijoh et al., 2003). The effect on endoderm morphology and differentiation can be assayed for endoderm markers and immunohistochemical analysis for epithelial polarity as reported earlier (Saund et al., 2012).

References

Adachi, H., Saijoh, Y., Mochida, K., Ohishi, S., Hashiguchi, H., Hirao, A. and Hamada, H. (1999). Determination of left/right asymmetric expression of nodal by a left side-specific enhancer with sequence similarity to a lefty-2 enhancer. *Genes Dev* **13**(12), 1589-600.

Banks, E. A., Toloue, M. M., Shi, Q., Zhou, Z. J., Liu, J., Nicholson, B. J. and Jiang, J. X. (2009). Connexin mutation that causes dominant congenital cataracts inhibits gap junctions, but not hemichannels, in a dominant negative manner. *J Cell Sci* **122**(Pt 3), 378-88.

Beahm, D. L., Oshima, A., Gaietta, G. M., Hand, G. M., Smock, A. E., Zucker, S. N., Toloue, M. M., Chandrasekhar, A., Nicholson, B. J. and Sosinsky, G. E. (2006). Mutation of a conserved threonine in the third transmembrane helix of alpha- and beta-connexins creates a dominant-negative closed gap junction channel. *J Biol Chem* **281**(12), 7994-8009.

Ben-Haim, N., Lu, C., Guzman-Ayala, M., Pescatore, L., Mesnard, D., Bischofberger, M., Naef, F., Robertson, E. J. and Constam, D. B. (2006). The nodal precursor acting via activin receptors induces mesoderm by maintaining a source of its convertases and BMP4. *Dev Cell* **11**(3), 313-23.

Dobrowolski, R., Sasse, P., Schrickel, J. W., Watkins, M., Kim, J. S., Rackauskas, M., Troatz, C., Ghanem, A., Tiemann, K., Degen, J. et al. (2008). The conditional connexin43G138R mouse mutant represents a new model of hereditary oculodentodigital dysplasia in humans. *Hum Mol Genet* **17**(4), 539-54.

Feistel, K. and Blum, M. (2008). Gap junctions relay FGF8-mediated right-sided repression of Nodal in rabbit. *Dev Dyn* **237**(12), 3516-27.

Francescatto, L., Rothschild, S. C., Myers, A. L. and Tombes, R. M. (2010). The activation of membrane targeted CaMK-II in the zebrafish Kupffer's vesicle is required for left-right asymmetry. *Development* **137**(16), 2753-62.

Hadjantonakis, A. K., Pisano, E. and Papaioannou, V. E. (2008). Tbx6 regulates left/right patterning in mouse embryos through effects on nodal cilia and perinodal signaling. *PLoS ONE* **3**(6), e2511.

Hatler, J. M., Essner, J. J. and Johnson, R. G. (2009). A gap junction connexin is required in the vertebrate left-right organizer. *Dev Biol* **336**(2), 183-91.

Kawasumi, A., Nakamura, T., Iwai, N., Yashiro, K., Saijoh, Y., Belo, J. A., Shiratori, H. and Hamada, H. (2011). Left-right asymmetry in the level of active Nodal protein produced in the node is translated into left-right asymmetry in the lateral plate of mouse embryos. *Dev Biol* **353**(2), 321-30.

Levin, M. and Mercola, M. (1998). Gap junctions are involved in the early generation of left-right asymmetry. *Dev Biol* **203**(1), 90-105.

Levin, M. and Mercola, M. (1999). Gap junction-mediated transfer of left-right patterning signals in the early chick blastoderm is upstream of Shh asymmetry in the node. *Development* **126**(21), 4703-14.

Liu, Y., Asakura, M., Inoue, H., Nakamura, T., Sano, M., Niu, Z., Chen, M., Schwartz, R. J. and Schneider, M. D. (2007). Sox17 is essential for the specification of cardiac mesoderm in embryonic stem cells. *Proc Natl Acad Sci USA* **104**(10), 3859-64.

Marques, S., Borges, A. C., Silva, A. C., Freitas, S., Cordenonsi, M. and Belo, J. A. (2004). The activity of the Nodal antagonist Cerl-2 in the mouse node is required for correct L/R body axis. *Genes Dev* **18**(19), 2342-7.

Meno, C., Gritsman, K., Ohishi. S., Ohfuji, Y., Heckscher, E., Mochida, K., Shimono. A., Kondoh, H., Talbot, W. S., Robertson, E. J., Schier, A. F. and Hamada, H. (1999). Mouse Lefty2 and zebrafish antivin are feedback inhibitors of nodal signaling during vertebrate gastrulation. *Mol Cell.* **4**(3), 287-98.

McGrath, J., Somlo, S., Makova, S., Tian, X. and Brueckner, M. (2003). Two populations of node monocilia initiate left-right asymmetry in the mouse. *Cell* **114**(1), 61-73.

Mohamed, O. A., Clarke, H. J. and Dufort, D. (2004). Beta-catenin signaling marks the prospective site of primitive streak formation in the mouse embryo. *Dev Dyn* **231**(2), 416-24.

Norris, D. P. and Robertson, E. J. (1999). Asymmetric and node-specific nodal expression patterns are controlled by two distinct cis-acting regulatory elements. *Genes Dev* **13**(12), 1575-88.

Oki, S., Hashimoto, R., Okui, Y., Shen, M. M., Mekada, E., Otani, H., Saijoh, Y. and Hamada, H. (2007). Sulfated glycosaminoglycans are necessary for Nodal signal transmission from the node to the left lateral plate in the mouse embryo. *Development* **134**(21), 3893-904.

Saijoh, Y., Adachi, H., Mochida, K., Ohishi, S., Hirao, A. and Hamada, H. (1999). Distinct transcriptional regulatory mechanisms underlie left-right asymmetric expression of lefty-1 and lefty-2. *Genes Dev* **13**(3), 259-69.

Saijoh, Y., Oki, S., Ohishi, S. and Hamada, H. (2003). Left-right patterning of the mouse lateral plate requires nodal produced in the node. *Dev Biol* **256**(1), 160-72.

Sarmah, B., Latimer, A. J., Appel, B. and Wente, S. R. (2005). Inositol polyphosphates regulate zebrafish left-right asymmetry. *Dev Cell* **9**(1), 133-45.

Saund, R. S., Kanai-Azuma, M., Kanai, Y., Kim, I., Lucero, M. T. and Saijoh, Y. (2012). Gut endoderm is involved in the transfer of left-right asymmetry from the node to the lateral plate mesoderm in the mouse embryo. *Development* **139**(13), 2426-35.

Schier, A. F. and Shen, M. M. (2000). Nodal signalling in vertebrate development. *Nature* **403**(6768), 385-9.

Sinner, D., Rankin, S., Lee, M. and Zorn, A. M. (2004). Sox17 and beta-catenin cooperate to regulate the transcription of endodermal genes. *Development* **131**(13), 3069-80.

Song, H., Hu, J., Chen, W., Elliott, G., Andre, P., Gao, B. and Yang, Y. (2010). Planar cell polarity breaks bilateral symmetry by controlling ciliary positioning. *Nature* **466**(7304), 378-82.

Tabin, C. J. and Vogan, K. J. (2003). A two-cilia model for vertebrate left-right axis specification. *Genes Dev* **1**;17(1), 1-6.

Takao, D., Nemoto, T., Abe, T., Kiyonari, H., Kajiura-Kobayashi, H., Shiratori, H. and Nonaka, S. (2013). Asymmetric distribution of dynamic calcium signals in the node of mouse embryo during left-right axis formation. *Dev Biol.* **376**(1), 23-30.

Tanaka, Y., Okada, Y. and Hirokawa, N. (2005). FGF-induced vesicular release of Sonic Hedgehog and retinoic acid in leftward nodal flow is critical for left-right determination. *Nature* **435**(7039), 172-7.

Thisse, C. and Thisse, B. (1999). Antivin, a novel and divergent member of the TGFbeta superfamily, negatively regulates mesoderm induction. *Development* **126**(2), 229-40.

Viotti, M., Niu, L., Shi, S. H. and Hadjantonakis, A. K. (2012). Role of the gut endoderm in relaying left-right patterning in mice. *PLoS Biol* **10**(3), e1001276.

Yoshiba, S., Shiratori, H., Kuo, I. Y., Kawasumi, A., Shinohara, K., Nonaka, S., Asai, Y., Sasaki, G., Belo, J. A., Sasaki, H. et al. (2012). Cilia at the node of mouse embryos sense fluid flow for left-right determination via Pkd2. *Science* **338**(6104), 226-31.